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STEM CELLS AND MINERALIZED TISSUE - CHARACTERIZATION AND DISEASE MODELING

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Till min familj,

Min man Shaho, mina föräldrar Favzieh och Ali, och min lillasyster Hanna

ABSTRACT

Deficits in mineralized tissue, as a result of abnormal development, trauma, disease or aging, are major medical problems. Consequently, new methods to regenerate stable and functional bone is a main focus in the field of tissue engineering. Cell therapy using stem cells that adopt a mineralizing phenotype holds great potential for regeneration of calcified tissues such as bone and teeth. Stem cells provide an unlimited number of cells that can be used for bone differentiation. The mechanisms governing stem cell bone formation are however complex, and involve many factors. In this context, knowledge of basic bone biology, handling of stem cells in *in vitro* culture systems and the complex molecular mechanisms associated with normal and impaired bone development is fundamental. The aim of this project is to enhance the understanding of stem cell differentiation into the osteoblastic/mineralizing lineage.

In the first study we investigated the roles of some of the constituents of the extracellular matrix (ECM) of dentin in mouse teeth. Using structural techniques, we found that osteoadherin (OSAD), a member of the family of mineralization-related small leucine-rich proteoglycan (SLRP) proteins, was localized at the mineralization front, closely associated with collagen fibers. This, together with data obtained from a functional assay, emphasizes the importance of OSAD in bone matrix maturation and mineralization. Continuing with *in vitro* model systems for subsequent application in the field of stem cells/regenerative medicine of bone and dentin, we highlighted important aspects of stem cells handling in the laboratory. We found that there were neither epigenetic alternations in selected histone modifications or marks nor any changes in protein expression when different passaging techniques were used. However, gene expression was significantly decreased for pluripotent markers using enzymatic split with a ROCK inhibitor, an effect that could be reversed upon mechanical passaging. These findings underline the fact that passaging techniques have to be taken into account when comparisons are made between cells that have undergone various treatments under different experimental conditions. In the last study we have derived induced Pluripotent Stem Cells (iPSCs) from a family with a mutation in the PIGT gene, which in addition to severe CNS defects causes a number of craniofacial bone and tooth abnormalities. iPSC-based models have emerged as useful systems to model human disease, both to unravel disease mechanisms and to provide test assays for drugs. We determined transcriptional and epigenetic changes in the patient-derived cells as compared to healthy control cells, with a focus on gene networks associated with bone development. We found four important genes to be downregulated compared to health unrelated iPSC controls, *OPN*, *MMP2*, *ACVR1*, and *MMP2* which all have shown to have important roles in in bone and skeletal development. Furthermore, they showed patterns of epigenetic regulation, highlighting the importance of histone modifications and DNA methylation in the disease.
Regenerative therapy with autologous cells using the patients' own reprogrammed cells to replace damaged tissue may reduce patient suffering and healthcare costs. This thesis presents findings that might be of future use in the development of such cell-based bone replacement strategies.

LIST OF SCIENTIFIC PAPERS

Hero Nikdin, Marie-Louise Olsson, Kjell Hultenby, Rachael Sugars. Osteoadherin accumulates in the predentin towards the mineralization front in the developing tooth.
PLoS One. 2012;7(2):e31525

Frida Holm, **Hero Nikdin**, Ros Kjartansdóttir, Giulia Gaudenzi, Kaj Fried, Ola Hermanson, Rosita Bergström-Tengzelius.
Passaging techniques and ROCK inhibitor exerts reversible effects on morphology and pluripotency marker gene expression of human embryonic stem cell lines.
Stem Cells Dev. 2013 Jul 1;22(13):1883-92

Hero Nikdin, Yaser Heshmati, Indranil Sinha, Malin Kvarnung, Magnus Nordenskjöld, Kelly Day, Mastoureh Shahsavani, Anna Falk, Kaj Fried, Julian Walfridsson.
Genome-wide characterization of human PIG-T mutant induced pluripotent stem cells.
Manuscript.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Mei Ling Lim, Philipp Jungebluth, Sebastian Sjöqvist, **Hero Nikdin**, Ros Kjartansdóttir, Christian Unger, Ivan Vassliev, Paolo Macchiarini
Decellularized feeders: an optimized method for culturing pluripotent cells
Stem Cells Transl Med. 2013 Dec;2(12):975-82

CONTENTS

LIST OF ABBREVIATIONS

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ACVR1	Activin receptor type 1
bFGF	basic fibroblast growth factor
BGN	Biglycan
BMC	Basic multicellular unit
BMP	Bone morphogenetic protein
C4S	Chondroitin 4-sulphate
C6S	Chondroitin 6-sulphate
CHD	Chromodomain helicase DNA-binding
ChIP	Chromatin immunoprecipitation
Chip-seq	Chromatin immunoprecipitation sequencing
CSF-1	Colony stimulating factor 1
DAPI	4,6-diamino-2-phenylindole dihydrochloridine
DCN	Decorin
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase
DS	Dermatan sulphate
ECM	Extracellular matrix
ESC	Embryonic Stem Cells
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FMD	Fibromodulin
FOXO1	Forkhead Box Protein O1
GAG	Glycosaminoglycan
HA	Hyaluronan
HAT	Histone actetyltransferases
HDAC	Histone deacetyltransferases
hESC	Human embryonic stem cells
hFF	Human foreskin fibroblasts
HS	Heparan sulphate
ICC	Immunocytochemistry
ICM	Inner cell mass
IHC	Immunohistochemistry
INO80	Inositol requiring 80

iPSC	Induced pluripotent stem cells
ISWI	Imitation switch
KS	Keratan sulphate
MBD	Methyl CpG binding domain protein
MECP	Methyl binding domain protein
MeDIP	Methylated DNA immunoprecipitation
MeDIP-seq	Methylated DNA immunoprecipitation sequencing
MMP	Matrix metalloproteinases
MMP2	Matrix metalloproteinase 2
MSC	Mesenchymal stem cells
NCC	Neural Crest Cells
NCP	Non-collagenous proteins
NSG	Next generation sequencing
NuRD	Nucleosome Remodeling and histone deactylation co-repressor complex
OCN	Osteonectin
OPN	Osteopontin
OSAD	Osteoadherin
PcG	Polycomb-group proteins
PFA	Paraformaldehyde
PG	Proteoglycans
PHD	Plant homeodomain
PIG-T	Phosphatidylinositol-glycan biosynthesis class T
PRC2	Polycomb repressive complex
PSC	Pluripotent stem cells
q-PCR	Quantative real time PCR
RANKL	Receptor activator of NF-$_K$B
rDPC	Rat dental pulp cells
RNA-seq	RNA-sequencing
ROCK	Rho-associated coiled-coil kinase
SLRP	Small leucine rich proteoglycans
SR	Serum replacement
SWI/SNF	Switching defective/sucrose nonfermenting
TDA	Thymine DNA glycosylase
TNALP	Tissue-nonspecific alkaline phosphatase

1 INTRODUCTION

1.1 **BONE TISSUE**

The skeleton is composed of cartilage and bone. It provides support for organs and tissues, and functions as a mineral homeostasis organ by regulating levels of calcium, magnesium, and phosphate ions (1). Bone is composed of 70% minerals and 30% organic material. The organic components consist of roughly 2% cells, 8% collagen type I, and 10% of proteins including non-collagenous proteins and other proteins such as osteonectin. Bone is derived from three different embryonic lineages. Neural crest cells are responsible for the formation of the entire facial skeleton, aside from the parietal bones, which are of mesodermal lineage, and epithelial linings in the skull (2). Bones of the head and the torso are derived from the somite, a packed segmental mass of mesoderm in the early embryo, while the limb skeleton is derived from the early mesodermal lineage called lateral plate mesoderm. Early in embryonic development, cells from these different sites migrate to their destined location, and once present they condensate, and differentiate into osteoblasts or chondrocytes (3) by two different processes:

1. Intramembranous ossification (flat bones): here, cells proliferate and directly differentiate into osteoblasts which form an ossification center, without any previous production of cartilage. The osteoblasts start to secrete osteoid, i.e. woven bone, which is unmineralized bone matrix characterized by its random orientation of collagen fibers and an accumulation of osteoid. As the osteid begin to accumulate, organic matrix composed mainly of type I collagen is deposited, followed by calcium phosphate deposition in the matrix forming bone.
2. Endochondral bone formation (long bones): a process where mesenchymal cells are differentiated into chondrocytes that form a cartilaginous matrix scaffold which is later replaced by lamellar bone (4). After chondrocyte formation, the chondrocytes in central regions of the cartilage undergo hypertrophy and start to synthesize ECM such as collagen. Following collagen deposition, angiogenesis is initiated (5). The calcified tissue cecomes vascularized, and the cells that are required to transform the cartilage scaffold into bone (e.g.osteoblasts, osteoclasts, hematopoetic cells) are recruited from the vasculature.

1.1.1 Bone homeostasis

Throughout life, bone is continuously shaped, remodeled and repaired to maintain its structural properties and its role in mineral homeostasis. This occurs through two separate mechanisms: bone resorption and bone formation, coordinated by osteoclasts and osteoblasts respectively. Bone resorption can also be initiated after injury or lesions, which in turn leads to bone regeneration/repair. A balance is maintained which depends on osteoblasts to form new bone, and on osteoclasts to remove excess bone. This balance is tightly controlled and any disruption may lead to bone disease, such as e.g. osteoporosis.

1.1.2 Cells involved in bone homeostasis

Osteoclasts are the resorptive cells of bone, involved in formation of skeleton and regulation of bone mass. The multinucleated osteoclasts form through fusion of myeloid hematopoietic precursors in the bone marrow. The precursors of osteoclasts are released into the bloodstream from the bone marrow. They circulate until they are attracted to bone by factors secreted during bone resorption, and subsequently become fully differentiated osteoclasts (6). Factors involved in the recruitment of osteoclasts are CSF-1 (colony stimulating factor 1) and

receptor activator of NF-$_K$B (RANKL), which are required for osteoclast differentiation during normal and pathological bone remodeling (6).

Osteoblasts synthesize bone matrix, and regulate mineralization. Osteoblasts originate from mesenchymal stem cells, and they undergo four maturation phases: pre-osteoblasts are formed by mesenchymal stem cells upon specific stimuli and signals, but lack the ability to produce mineralized tissue. The pre-osteoblasts mature into osteoblasts, which are cuboidal in shape, have a strong alkaline phosphatase activity (indicative of mineral producing cells), secrete type I collagen and produce non-collagenous proteins (7). Osteoblasts are controlled by a master transcription factor named RUNX2 (runt-related transcription factor 2). Mice that lack RUNX2 have a skeleton mainly composed of cartilage and a decrease in mineralization as a consequence of arrest in osteoblast maturation (8, 9). There are different signaling pathways involved in osteoblastic bone formation. One of them is the Wnt/β-catenin pathway. Binding of Wnt to its receptors, Frizzled and the two co-receptors LRP5 and LRP6, leads to a complex signaling which results in the initiation of transcription genes involved in osteoblast differentiation, function and survival. Mutations in the LRP5 receptor causes deregulated bone formation leading to either loss or gain of bone in skeleton due to altered osteoblast function (10). The mature osteoblasts can differentiate into osteocytes, which are terminally differentiated osteoblasts woven into the bone matrix. They are smaller than osteoblasts and are believed to maintain the bone structure (11). Bone lining cells is another cell type formed from mature and differentiated osteoblasts. These cells are present at sites where bone surfaces are not remodeled, and are suggested to prevent inappropriate interaction of osteoclasts with bone (12).

1.1.3 Regulation of bone homeostasis

Bone remodeling occurs over several weeks and is governed by small aggregations of cells called basic multicellular unit (BMU). These include osteoclasts, osteoblasts, osteocytes (osteoblast origin) within the bone matrix, and the bone lining cells covering the bone surface. There are different steps in the remodeling cycle of bone (13-15) (Figure 1):

1. Initiation and activation phase: Firstly the osteoblasts respond to certain signals due to damage or hormone signals (such as estrogen or PTH) secreted by ostecytes within the bone. Chemokines are secreted by the osteoblasts, resulting in the recruitment of pre-osteoclast from the circulation to the site of remodeling. Pre-osteoclasts then differentitate into mature osteoclasts. Their activation and maintenance of bone resorption is controlled by RANKL and CSF-1 secreted from the osteoblasts, which increases osteoclast formation and activity.
2. Reversal/resorption phase: a transition phase in which osteoclasts undergo apoptosis, and osteoblasts are recruited and begin to differentiatie into their mature state. Matrix debris is removed by tissue macrophages, osteomacs, that produce MMPs to facilitate the process. The main purpose of this phase is to produce signals, including TGF-β and insulin-like growth factors, which will inactivate of bone resorption and allow activation of bone deposition.
3. Termination phase: Mesenchymal stem cells or pre-osteoblasts return to the resorption site, where they undergo terminal differentiation and form new bone. The last step in bone formation is hydroxyapatite incorporation into newly deposited bone.

The remodeling cycle is completed when an equal amount of resorbed bone has been replaced. Osteoclasts undergo apoptosis; the mature osteoblasts form bone-lining cells or differentiate into osteocytes. The balance is kept and maintained until next time remodeling is initiated.

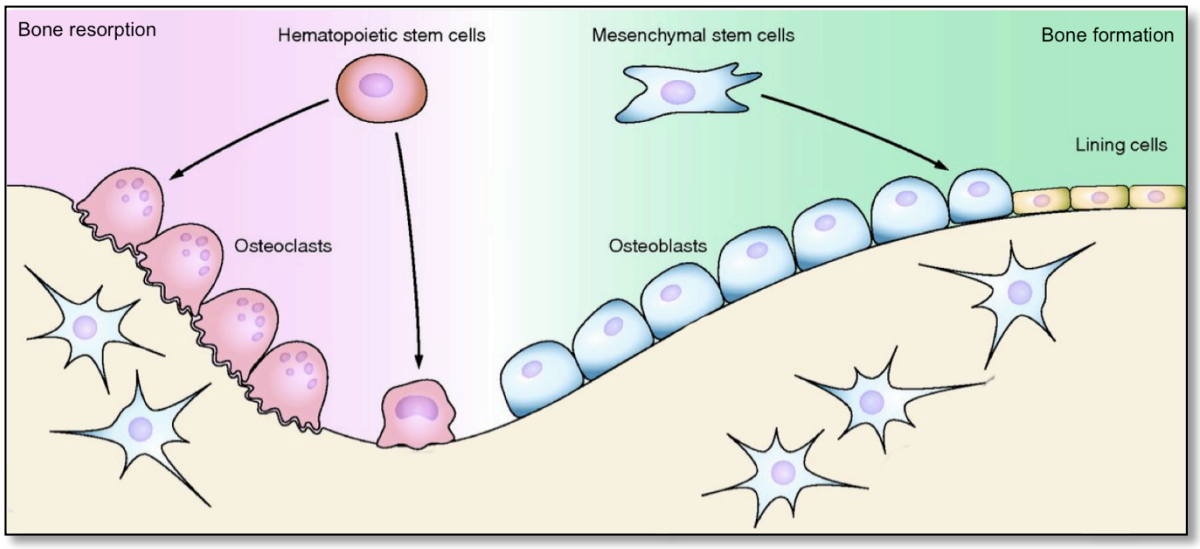

Figure 1. Skeletal formation involves bone remodeling and growth: Howship's Lacunae. *In the mature bone, bone tissue is constantly being remodeled by osteoclasts and osteoblasts. The osteoclasts resorb bone and form pits or cavities called Howship's Lacunae. These are attachment sites for osteoblasts, which will produce ECM proteins to fill the cavity with an unmineralized matrix named osteoid. The osteoblasts then mineralize the osteoid by laying down hydroxyapatite to complete the bone remodeling cycle. The equilibrium between osteoblasts and osteoclasts is crucial. Any shift in balance between them can cause a pathological condition. Picture adapted from (16).*

1.1.4 Stem cell models for osteogenesis

Bone has a considerable intrinsic regenerative capacity. However, after excessive bone loss through e.g. aging or disease, inherent bone regeneration is insufficient. Autologous bone graft techniques have been used as bone replacement therapy (17), but unfortunately it is associated with morbidity and pain at the affected site (17, 18). Autologous mesenchymal cells from the bone marrow constitute a promising source of cells for bone defect treatment, as they have the potential to differentiate into bone, cartilage and fat (19). The use of bone marrow mesenchymal stem cells in regenerative medicine is however somewhat hampered by the fact that they have a restrictive proliferative potential. In addition, they have a reduced proliferative capacity during aging (20, 21). ESCs have also been used for differentiation into osteoblasts. Using embryoid bodies, mouse ESC have been differentiated into osteoblasts *in vitro* and *in vivo*, and human ESC have been utilized for generation of osteoblasts *in vitro* (22, 23). Despite this, the use of human ESC faces major challenges. Thus, the ethical aspects concerning the derivation of ESC from human embryos are complex. Furthermore, ESCs may elicit immunogenic responses, as they are usually allogeneic.

As seen from above, it is of great importance to develop efficient *in vitro* culture techniques in order to obtain cells that can generate functional bone. In the field of regenerative

medicine, hiPSC have been used for differentiation into various cell types (23-26). iPSC have been differentiated into a mesenchymal phenotype and when further cultivated using different carriers such as e.g. silk scaffold (27), fibronectin-like engineered polymer protein (28), or a gelfoam matrix (29, 30) they had the potential to differentiate into the osteogenic lineage both *in vitro* and *in vivo* in mouse (30).

However, much work remains to determine which stimulating factors that are needed for iPSCs to differentiate into osteoblasts, and which appropriate time points, durations, and concentrations that are required. The protocol widely used for the induction of the osteoblastic lineage lists ascorbic acid (50μg/ml), β –glycerolphosphate (10nM) and the synthetic glucocorticoid dexamethasone (10nM or 100nM) (22, 31, 32). Dexamethasone is the most essential component and its supplementation is necessary for osteogenic differentiation, although concentrations and duration of supplementation has been shown to vary (31). Ascorbic acid is not essential for bone formation, but its addition to differentiating cell cultures increases the production of collagen rich EMC, and β–glycerolphosphate provides the right environment for mineralization by promoting calcium phosphate deposition (33). There are additional factors frequently used in osteogenic differentiation, such as a) 1, 25-dihydroxyvitamin D3 which in combination with dexamethasone accelerates cell growth, increases expression of alkaline phosphatase activity and osteogenic markers such as osteocalcin (34) and b) parathyroid hormone, which stimulates osteoblastic development and when further administered increases bone mass and density (35). In addition, bFGF2, and bone morphogenetic proteins (BMP) have been shown to have positive effects on bone formation *in vitro* (36). A sequential supplement of bFGF2 and BMP2 resulted in increased alkaline phosphatase activity and osteocalcin levels. bFGF2 was shown to be important for the early proliferation of immature osteoblasts bone matrix formation, and this was further stimulated by BMP2 addition later in the cultures (36).

When creating osteoblastic cells *in vitro,* different scaffolds are used that contain compounds, which do not occur at comparable levels *in vivo*. Later, at transplantation *in vivo,* these cells must be in an environment that holds specific factors and cell signals that promote their growth and osteogenic potential. Hence, there are a number of major issues in bone tissue regeneration that have to be considered These include the choice of cells for differentiation, the design of supporting scaffolds both *in vitro* and *in vivo*, the selection of growth factors to stimulate signaling pathways involved in maintenance of bone remodeling, and the subsequent maintenance of derived functional bone cells.

1.1.5 Epigenetics of bone formation

In vitro stem cell studies of how epigenetics control bone formation gives clues about how the *in vivo* mechanisms operate. Differentiation of MSC into osteoblasts was followed by a reduction in expression of stem cell related genes such as brachyury and LIN28 (37). This was shown to be mediated by methylation at the CpG islands promoter of these genes. Osteocalcin (OCN) is a NCP secreted by osteoblasts and has bone-forming capacity. In undifferentiated genes OCN has been shown to be hypermethylated and associated with condensed chromatin structure. Further, during *in vitro* stimulation of osteogenesis, the hypermethylated promoter of OCN is significantly decreased as the expression of OCN increases (38). High levels of acetylation at the OCN promoter regions during osteoblast development have also been shown (39), which in another study was suggested to be explained by decreased levels of HDAC (40). HDAC, the enzyme responsible for removing acetyl groups from histones and interactions with transcriptional repressors, was significantly decreased during osteoblast differentiation. Recruiting HDAC to the promoter sites of OCN

resulted in repressed differentiation of osteoblastic lineage (40). Additionally, HOXA10 is necessary in embryonic development and is involved in osteogenic termination by activation of RUNX2 and OCN. Activation of these genes is mediated through chromatin hyperacetylation and the recruitment of the active methylation mark H3K4me3 (41).

microRNAs (miRNA) also influence osteogenesis, both in embryonic development and during maintenance of mature bone tissue. To study the role of miRNA in bone development, specific disruption of the miRNA machinery has to be achieved. This can be done by deletion of Dicer which is required for the production of functionally mature miRNAs (42). Dicer deletion in the NCC results in deregulated morphogenesis and also deformities in skull development (43). Mice with a conditional deletion of Dicer in pre-osteoblasts showed an inhibition in the formation of a mineralized matrix, indicating a distinct role of miRNAs in early bone formation (44). miRNAs are also important in maintaining bone homeostasis. An absence of miRNA in preosteoclasts and in mature osteoclasts yields a phenotype with increased bone mass. This is due to decreased activity and reduced numbers of osteoclasts (45, 46).

1.1.6 Dentin as a model for bone mineralization

Bone and dentin consist of crystals of hydroxyapatite that are deposited in an oriented fashion on a matrix of collagen type I. The collagen molecule is a triple helix, and when presented to matrix molecules and minerals it becomes stiff and provides support. The mineral components align themselves along the axes of the collagen fibrils, and hydroxyapatite can be deposited. The tooth offers good model systems to study mineralization, since it is formed by different types of hard tissue (enamel, dentin, cementum) where each type has a specific degree of calcification. The major building blocks of dentin are mineralized matrix, collagen fibrils and a wide range of NCP, rendering it analogous with bone in terms of structure. This makes dentin an excellent model tissue where all aspects of biomineralization can be studied in detail from cellular compartments to mineralizing zones

1.1.7 Dentin matrix protein during mineralization

The vertebrate tooth is a complex structure made up of distinct soft and calcified tissue: the stromal pulp, the predentin, dentin, the enamel and the root cementum. Enamel is formed by specialized epithelial cells, which are ectoderm-derived, whereas dentin is formed by NCC-derived cells known as the odontoblasts. NCC is a small and transient population of multipotent cells arising from the folding neural tube very early in development. The craniofacial NCC contributes to an impressive diversity of cell types in addition to odontoblasts, including chondrocytes, osteoblasts, smooth muscle cells, melanocytes, adipocytes, neurons and Schwann cells (47, 48).

The ECM of skeleton and dentin are very similar. It is primarily made up of a 3D network of collagen fibers, where type I collagen (90%) is predominant, non-collagenous proteins (NCP) and crystals of hydroxyapatite (49, 50). Odontoblasts, which are very similar to osteoblasts of the skeleton with only some differences in gene expressions (51), are responsible for the synthesis of ECM. Common protein markers for bone and dentin include bone sialoprotein, RUNX2, and OSAD (52, 53). Dentinogenesis is believed to be controlled by the expression of ECM proteins of NCP called proteoglycans (PG). They undergo transformations and alterations from the unmineralized predentin to the mineralized dentin and also play a role in structural and metabolic functions in mineralized tissues (54, 55). A number of PG's have been identified in predentin and dentin of tooth, and several of them belong to the family of

SLRP (54, 56). SLRPs consist of 17 members of secreted proteins. 11 of these are listed in Table 1. They consist of two main structural domains, a conserved protein core and several glycosaminoglycan (GAG) side chains.

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1.1.8 Small leucine-rich proteoglycans

SLRPs have been identified in the predentin and dentin of teeth and in long bones. They have important roles in the mineralization process where they organize the collagen framework and interact with hydroxyapatite (54, 57). SLRPs are recognized as active components of the ECM and in the body they are found invariably in all soft and hard-mineralized tissues (58, 59). These cell-derived macromolecules are believed to control the mineral deposition and thus have a role in dentinogenesis and bone.

SLRP's share a common structure consisting of:

1. The amino-terminal domain, containing the negatively charged GAG chains. To date 7 GAG chains have been described: chondroitin 4-sulphate (C4S), chondroitin 6-sulphate (C6S), dermatan sulphate (DS), hyaluronan (HA), heparan sulphate (HS), heparin and keratan sulphate (KS) (54). This region is supposed to be involved in binding to ECM proteins and other cell surfaces (60).
2. The leucine-rich repeat: The structural motif of the leucine-rich repeat is a distinct hallmark, comprising 80% of the protein. It has 10 to 11 repeats of 20-29 amino acids, is flanked by cysteine motifs on both the N- and C terminal, and participates in a wide range of biological functions. It provides most of the biological functions such as interaction with the triple helical collagen, but also controls collagen fibril diameter (55, 61, 62).
3. The carboxyl end domain which is flanked by two cysteine residues. Its function is still not well understood.

1.1.9 Osteoadherin – a keratan sulphate SLRP

OSAD is a keratin sulphate SLRP that belongs to the class II family of PG. It was identified and purified from the mineral content of bovine long bone by Wendel *et al* (63). It has a molecular weight of 47kDa, and its expression is specifically localized to mineralized tissues (53, 63). It has a strong integrin-dependent cell-binding ability (through $\alpha v\beta 3$) and a high affinity for hydroxyapatite. It is an initial attachment site for osteoblasts and has been postulated to bind to the triple helical collagen; furthermore OSAD mRNA is strongly expressed in mature osteoblasts (63). The expression of OSAD in mineralized bone matrix resembles that of the osteoblast marker bone sialoprotein (BSP) (64), suggesting that OSAD, could be used as an additional mineralization-specific cellular marker. Further underlining its role in mineralization, OSAD has high affinity for hydroxyapatite considering its acidic properties via the rather large and acidic C-terminal, which consists of 69 amino-acids (57, 63, 65). In line with gene expression studies, OSAD protein is deposited in mineralized

matrix during alveolar bone formation. Furthermore, OSAD is also expressed during development of the rat molar tooth, and has been suggested to be involved in dentinogenesis (66, 67).

1.1.10 Fibromodulin – a keratan sulphate SLRP

FMD is a 59 kDa keratin sulphate SLRP that belongs to the class II family of PG. It was first identified in cartilage, skin and cornea (59, 68). Similar to other SLRP's, FMD is also involved in aggregation and orientation of collagen fibrils (69), and FMD deficient mice have thinner collagen fibrils (70). Further, FMD is thought to have an active role in mineralization and dentinogenesis. Keratan sulphate SLRP's show an increased gradient in expression from proximal to distal parts of the predentin, where the mineralization occurs. FMD has also been detected in the terminal parts of odontoblasts and in the predentin (71, 72). FMD deficiency leads to hypomineralization in dentin, as a consequence of increased diameter and irregular patterns of the collagen fibrils in predentin. FMD and Biglycan (BGN, see below) both inhibit collagen fibrillogenesis in predentin, and they also display the same distribution patterns in predentin (58). The importance in these SLRP's in bone development was demonstrated in in FMD and BGN double knockout mice. These mice displayed gait impairments, ectopic ossification centers and severe premature osteoarthritis Analysis of collagen fibrils showed significant changes in distribution, size and diameter compared to the wild type (73). Together, these data suggest that FMD might play an important role in dentin and enamel formation.

1.1.11 Decorin and Biglycan – chondroitin/dermatan sulphate SLRP

DCN and BGN are both small chondroitin/dermatan sulphate extracellular matrix molecules belonging to the class I family of PG. They are ubiquitously expressed in connective tissues and both have important biological functional roles mediated by interactions with ECM proteins and cytokines. In addition, both are important regulators of collagen fibrillogenesis I (59). DCN and BGN compete, and bind to the same site of the collagen fibrils, whereas FMD binds to a site distinct from this locus (74). DCN-deficient mice are viable but have fragile skin with reduced tensile strength, due to the irregular patterns and smaller number of collagen fibrils, as well as increased collagen diameters (75). Similar patterns of irregular and abnormal fibril structures can be seen in the BGN deficient mouse (76, 77). BGN is highly expressed in bone and believed to be involved in skeletal growth. Newborn BGN deficient mice had no obvious phenotype, but subsequent growth was reduced and a couple of months postnatally their bone mass was reduced when compared to wild-type animals (78). Thus BGN seems to be an important molecule both in the positive regulation of bone formation and bone mass and in collagen fibril formation. The DCN and BGN deficient mice both showed severe and complex disruptions in dentinogenesis. The most prominent feature in BGN-deficient mice was a massive deposition of enamel and acceleration of enamel formation. In contrast, in DCN deficient mice the dentin was severely hypomineralized and enamel formation was delayed (72). These changes have been observed in animal models, but to date there have been no reports on defects in human teeth or bone caused by DCN or BGN gene mutations.

Table 1.

SLRP Member	Class	Number of LRR	GAG type/member	Deficiency	References
Biglycan	I	12	CS/DS	Reduced bone mass	(77, 78)
Decorin				Fragile skin	(68, 70)
Fibromodulin	II	12	KS	Defects in collagen fibrillogenesis	(59, 71)
Osteoadherin				N/A	(57, 67)
Opticin	III	8	KS	N/A	(79)
Epiphycan			DS	N/A	(80)
Chondroadherin	IV	12	Not examined	N/A	(81)
Tsukushi					(82)
Nyctalopin					
Podocan	V	20	Not examined	N/A	(83)
Podocan-like 1 protein					(84)

1.1.12 Predentin – an active site for mineralization

The predentin compartment offers a great advantage over bone for studies of mineralization, as the mineralization process occurs only in one region and also because the hard tissue of the tooth does not undergo remodeling in the way that bone does. The initially secreted proteoglycans and GAGs act as inhibitors of mineralization but facilitate the organization of the extracellular matrix for subsequent mineralization. Biochemical, histochemical and ultrastructural studies have shown that SLRPs and GAG chains are differently distributed across the mineralizing tooth from the odontoblast cell layer to predentin, predentin/dentin interface and dentin (52, 54, 58, 85). The predentin has been shown to be a very active area of mineralization, where NCP such as SLRPs and different matrix metalloproteinases (MMP) are very important as vehicles that transport collagen, proteoglycans and NCP and reorganize the matrix (49). Across this gradient, the GAG chains become more associated with collagen

fibers, allowing a more defined fibrillogenesis to take place (86). The reduction in GAG chains is a result of MMP's that cleave the core protein and thus liberate a proportion of the GAG chains. In summary, the major roles of SLRPs in dentinogenesis are to control collagen fibrillogenesis, to bind calcium, to interact with hydroxyapatite and also to inhibit crystal growth (52, 54, 59, 72).

1.2 STEM CELLS

1.2.1 Human embryonic stem cells

Embryonic stem cells (ESC) are derived from totipotent cells in the mammalian embryo and are capable of contributing to all germ layers in the body and the germ line (87). This is in contrast to multipotent/adult stem cells, such as hematopoietic stem cells, which have a controlled differentiation capacity. ESC can expand unlimitedly, and remain undifferentiated *in vitro* (88). In 1981, the first ESC line was derived from mouse blastocyst, and propagated *in vitro* (89). It took another 17 years until the first human ESC (hESC) line was derived from the inner cell mass of embryos (88). hESC are used in the laboratory for researchers to understand normal development and disease, and particularly since the provide putative tools for cell-based therapies in regenerative medicine. However, their use is not without problems:

i. Ethical barriers hinder the full usage of these cells in therapies
ii. As these cells are not patient-specific, they may cause unwanted immunogenic responses

1.2.2 Human iPSC

In 2006 Takahashi and Yamanka introduced iPSC. The discovery that the introduction of only four factors into differentiated somatic cells was enough to reprogram them into pluripotent cells changed the stem cell field. This was achieved first in mouse (90) by retroviral overexpression of a set of four transcription factors: Oct4, Sox2, Klf4, c-MYC or Lin28 and Nanog (91), and later in human fibroblasts (91, 92). The use of iPSC created from a patient's owns somatic cells eliminate the ethical as well as the immunogenic problems associated with hESC. hiPSC can tentatively become valuable for future cell-based therapy and forward the field of regenerative medicine Figure 2.

After the discovery of reprogramming, there has been extensive effort in redesigning the method for delivery of the reprogramming factors. The main focus has been to increase the efficiency and to remove or decrease the vector integration into the host genome. The reprogramming efficiency of the first mouse iPSC was only 0.02% (90). In hiPSC, the efficiency has reached 0.1% (92). Lentivirus based approaches, and replacing c-MYC with Lin28 has lead to a transduction efficiency of between 0.03-0.05% (91). In addition to the integrating viruses, there are many other ways in which somatic cells can be reprogrammed:

1. Nonintegrating viruses; adenoviruses (0,0001% efficiency) (93), sendai virus (0.1%) (94) and protein delivery 0.001% (95).
2. Nonviral; mRNA (4.4%) (96), miRNA (0.002% - 0.2%) (97), piggybac (0.02%-0.05%) (98).
3. Somatic cell nuclear transfer: enucleated oocytes are implanted with a nucleus from a somatic cell (efficiency approximately 100%) (99).

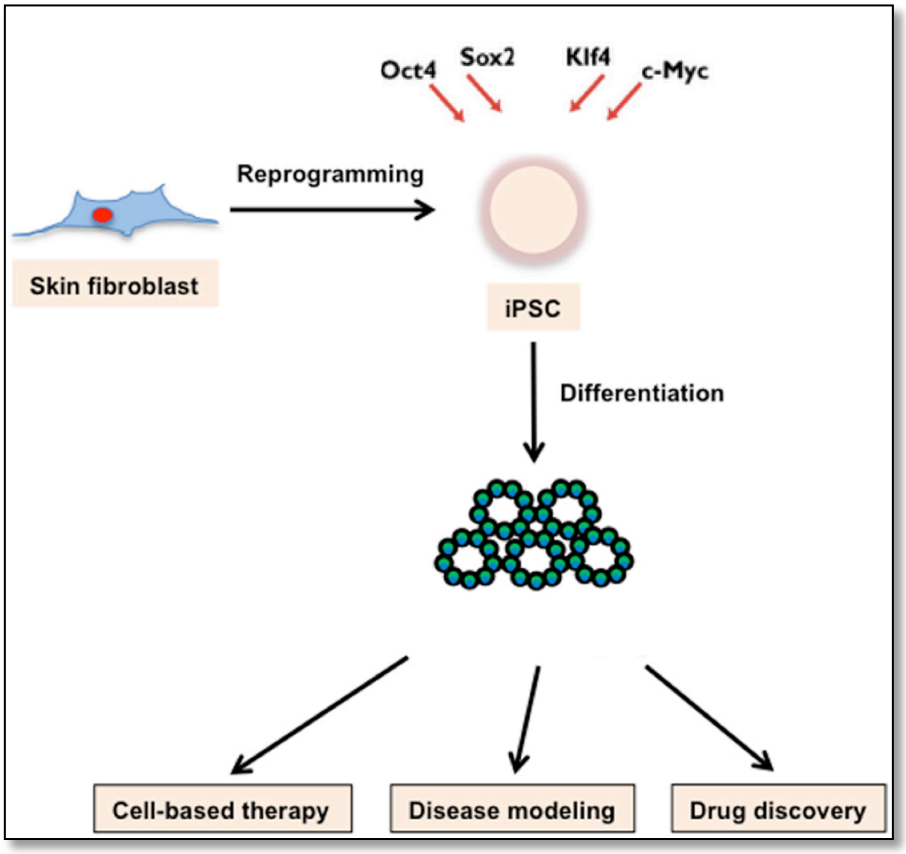

Figure 2. Induced pluripotent stem cell

Somatic cells can be reprogrammed to a pluripotent stage by the ectopic co-expression of transcription factors. The reprogrammed cells can then be differentiated into any desired cell type and used in cell-based therapy, disease modeling or for drug discovery. Picture adapted from (100).

Inefficiency is a great limitation when reprogramming somatic cells into iPSC. Thus, the derivation of iPSC from patients is still time-consuming, expensive and laborious. The need for more robust techniques is required, and perhaps there is a need of complementing reprogramming methods with other tools. It has been shown that the methyl CpG binding domain protein (MBD3), which regulates chromatin, is a hinder for reprogramming. MBD3 is a subunit of the nucleosome remodeling and histone deactylation co-repressor complex (NuRD). When the levels of MBD3 were decreased or abolished, the reprogramming efficiency reached 100%, and iPSC clones could be seen within 7 days (101). This finding is however questioned, as ESC lacking MBD3 has been shown to be viable but display a continued self-renewal phenotype, and lack lineage commitment, suggesting a requirement for MBD3 in ESC (102). Furthermore, the complete removal of MBD3 was shown to impair the generation of iPSC from neural stem cells, and overexpression of MBD3 resulted in higher levels of pre-iPSC (103). This was suggested to be due to higher levels of MBD3/NuRD, playing a positive role in the reprogramming process.

1.2.3 Derivation of hiPSC – Sendai virus mediated reprogramming

Sendai virus vector causes an efficient transfection and persistent expression of the transcription factors Oct4, Sox2, Klf4, and c-Myc, but does not integrate into the host

genome. The replication occurs in the form of negative-sense single stranded RNA in the cytoplasm. Negative-sense strand RNA does not encode for mRNA and must be translated to a sense strand RNA before it can be translated into a functional protein. Sendai virus replicates independently of the cell cycle, causing no genetic changes transferred to daughter cells. In other methods where e.g. retroviruses and lentiviruses are used, the genome of the virus is converted to DNA in the infected cells and integrates into the chromosome. It is then transcribed and expressed as any other gene (104). Sendai virus has been used to reprogram human fibroblasts with an efficiency of 0.1% (105). As there is no risk of Sendai viruses being integrated into the genome, this virus-based vector was shown to be safe for gene therapy (106). This feature prevents pluripotency factors to be actively transcribed along with the host genes, making it a better candidate for clinical applications, compound screening, or disease pathways studies. Furthermore the genes introduced with Sendai virus are decreased after iPSC establishment and further decreased over time (105). This occurs in the integrating viruses as well, but the advantage of Sendai virus over integrating viruses is that it does not induce a lesion in the genome with associated risks of mutations. Such mutagenic lesions are often related to tumorigenic and cancerous outcomes.

1.2.4 Maintenance of undifferentiated hiPSC

Undifferentiated pluripotent cells have distinct cell morphology. The colonies of cells grown on feeder layer have large nuclei and distinct nucleoli, and the cells are tightly packed with tight and sharp borders (Figure 3). Differentiated cells lose these features. The membranes may become irregular and indistinct, while colonies might be enlarged, more flattened in appearance and stacked upon each other creating a thick surface. One of the difficulties in culturing hiPSC is that the culture conditions are not always fully xeno-free or chemically defined. The ideal culture method for hiPSC in clinical applications and regenerative medicine would be a combination of animal-free, serum-free and also a feeder-free culture system. hiPSC are maintained under the same culture conditions used for hESC (107) and thus cultures of hiPSC and hESC are treated in the same way. The first lines of hESC were cultured on mitotically inactivated fetal mouse fibroblasts. The feeder cells have dual roles in maintaining stem cells. They act as a matrix but also as a supportive layer and provide the hESC with factors necessary for their propagation. Removal of the feeder layer leads to unwanted differentiation (108), suggesting that the feeder's layers secrete factors necessary for pluripotency, including bFGF2 and activinA (109). Other cell matrices have been used such as Matrigel®, derived from a mouse sarcoma tissue (110), laminin, fibronection, and Matrigel® in combination with conditioned mouse feeder cell media (111, 112). The use of a non-human cell scaffold for hiPSC entails the risk of transmitting animal related pathogens to cells which then will be unsuitable for clinical use. Additionally, the use of feeder free matrices might present problems of batch-to-batch variations. Using either a human supportive cell line, hFF, which has been irradiated (113), or human so called serum replacement (114) appears to overcome the hurdle of employing cell matrices or products derived from animals. However, culture conditions using feeder layers is laborious and time consuming. Cells cultured under these conditions cannot supply the need for large number of cells needed for basic research and potential clinical applications. In 2014 laminin-521 was introduced as a substrate for hiPSC and hESC, and they could now be grown under xeno-free and chemically defined conditions (115).

The long-term stability of hiPSC is important and depends on a growth medium that supports the undifferentiated state of the cells. The typical medium contains KnockOut Dulbecco's modified Eagle's medium, supplemented with 20% KnockOut Serum Replacement, 2 mM

glutamax, penicillin streptomycin 0.5%, 1% nonessential amino acids, 0.5 mM 2- β_2-mercaptoethanol, and various concentrations of bFGF2 (116, 117). bFGF2 is important for the cells to remain pluripotent and is thus essential for culture medium, and allows for the continued growth of hiPSC (116). bFGF2 can interrupt BMP signaling, leading to inhibition of trophoblast (111), and endoderm differentiation (118). Commercially available medium such as mTeSR™1 or 2 and NutriStem® can be used when the cells are grown on feeder free matrices such as Matrigel®.

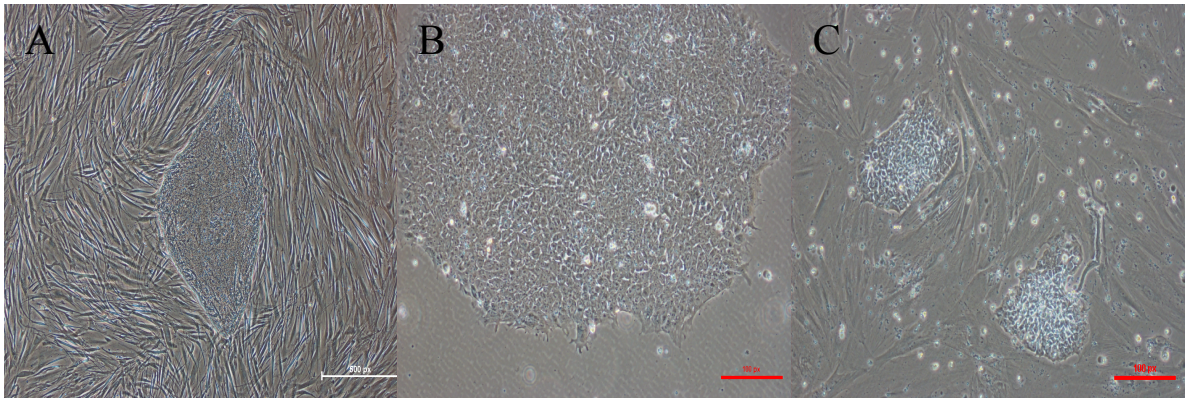

Figure 3. Cell morphology during passaging, on feeder plates and Matrigel®.

PSC have smooth and sharp edges. (3A) iPSC line grown on hFF plates and split mechanically shows typical characteristics of PSC (3B-C) hESC line, hES360, grown under feeder free conditions using Matrigel® plates. (3B) is split mechanically and (3C) is split enzymatically. Both conditions display the typical characteristics of PSC, smooth and sharp edges.

1.2.5 Regulation of molecular circuitry in hiPSC

PSC are under tight control of a transcriptional machinery, including transcription factors required for reprogramming, chromatin modifying enzymes, regulatory RNA molecules (such as miRNA), DNA methylation systems, and different histone marks (119). This machinery allows for the accessibility of DNA during pluripotency and cells in a differentiated state. The transcription factors Oct4/POU51, Sox2, and Nanog are crucial regulators of PSC identity and are expressed in both PSC and ICM of the blastocyst (120-122). Disruption of Oct4 or Nanog leads to differentiation of PSC and ICM to extra-embryonic endoderm and trophectoderm, respectively. These transcriptional factors co-occupy promoters sites of many genes, genes that are of importance in the circuitry involved in maintaining PSC (123).

Oct-3/4, a POU transcription factor encoded by the gene POU5f1, is a master regulator of pluripotency. Oct4 is believed to lock pluripotency, suggesting that it is a gatekeeper preventing differentiation into trophectoderm (124). Levels of Oct4 are always in balance, an increase of less than two fold causes differentiation into primitive endoderm and mesoderm, and repression results in loss of pluripotency and differentiation into trophectoderm (124). Oct4 occupies 3% of promoters of known protein-coding genes in hESC, including the genes encoding for Sox2, Nanog, and other genes with known high transcripts in ESC (123).

Approximately half of the promoters occupied by Oct4 are also co-occupied by Sox2 (123). Nanog was first discovered when it was shown that it could maintain the self-renewal of mouse ESC, without the presence of cytokines required for pluripotency (120). Disruption of Nanog yielded increased expression of differentiation markers, but expression of Oct4 was maintained (121). Furthermore, Nanog was also suggested to be necessary for the derivation of ESC, as the ICM of mouse ESC did not have the ability to remain undifferentiated *in vivo* (121). Surprisingly, Nanog was not among the factors required for reprogramming of mouse somatic cells to a pluripotent state (90, 125), but was included in reprogramming of human somatic cells (91). However, in combination with Oct4, Nanog was used to characterize true iPSC populations (126, 127). Overall, Nanog is essential in establishing ICM *in vivo* and generate a bona fide iPSC population, but appears to be dispensable for initial introduction of reprogramming (126, 128). Sox2 is a member of the SRY-related HMG box gene family. It is crucial for maintaining pluripotency of ESC, and for regulating the expression of Oct4 (129). Inhibition of Sox2 leads to differentiation of ESC into multiple cell lineages, and deletion of Sox2 leads to trophectoderm and a failure to maintain pluripotency. Furthermore, a down-regulation of Sox2 leads to down-regulation of Oct4 activators and up-regulation of Oct4 repressors (130).

Oct4, Sox2, and Nanog are believed to function as an auto-regulatory group, by affecting genes important in PSC. All three bind to their own promoters but also to the promoters of genes of each other. More than 90% of promoter regions bound by Oct4 and Sox2 were also co-occupied by Nanog. Furthermore all three showed close proximity to all genes that they occupied together (123). This auto-regulatory mechanism is believed to improve and increase gene expression and maintenance, thereby facilitating pluripotency Figure 4.

1.2.6 Signaling transduction pathways

Apart from the transcription factors, human PSC are under tight control of different signaling pathways.

1. MAPK-ERK pathway: high levels of activity in undifferentiated cells, where its ativation is related to bFGF signalling and helps the cells to remain in an undifferentiated state (131).
2. PI3K pathway: this pathway is important in proliferation, maintenance and survival of PSC (131). Inhibition of PI3K leads to differentiation in hESC, and activation of this pathways is suggested to be stimulated by growth factors such as bFGF.
3. Wnt signalling pathway: Activation of Wnt pathway can maintain PSC in an undifferentiated state and also maintain expression of pluripotency markers. This signalling pathway is endogenously activated in ESC and has been shown to be downregulated when cells are differentiated (132).
4. TGFβ pathway: TGFβ is a member of a large family of growth and differentiation factors, including activin, nodal, and bone morphogenetic proteins (BMP). In mouse ESC BMP4 maintains self-renewal, but in hESC it induces tropoblast and endoderm differentiation (133). Noggin is an inhibitor of BMP4 and aids to maintain the undifferentiated state in hESC (134).
5. bFGF pathway: bFGF is a key signalling pathway responsible for keeping human PSC undifferentiated. Receptors of FGF signalling are highly enriched in undifferentiated cells, as compared to their differentiated counterparts, and amongst the four receptors, FGF receptor 1 is most commonly seen in undifferentiated ESC. The concentration of bFGF is dependent on what cell matrices that are used for culturing. Normally, the concentration ranges between 4ng/ml and 8, 40 or 100ng/ml

(134, 135). Many theories on why bFGF supports growth of undifferentiated cells have been proposed, including that bFGF might inhibit BMP activity (134).

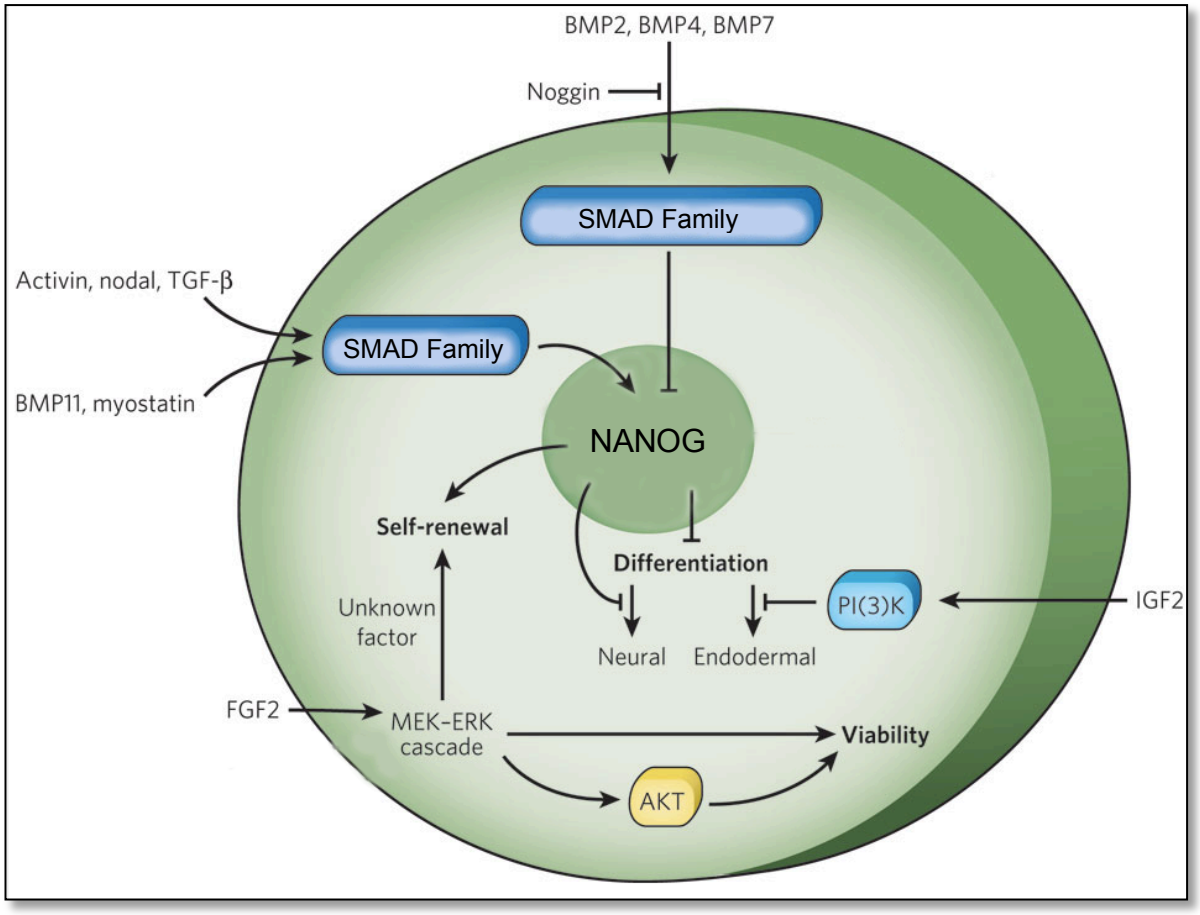

Figure 4. Complex signal transduction circuitry in PSC.

There is a complex signal transduction cascade involved in maintaining PSC. The ligands for TGFβ and bFGF activate membrane-bound receptors and signals into the nucleus to the core of the PSC transcription factors. Signallning from TGFβ family, including BMP, activin, and nodal act mainly on Nanog, which aids in self-renewal of PSC and keeps them undifferentiated, whereas the MEK-ERK cascade inhibits apoptosis and anoikis. The PI3K pathway hinders PSC from differentiating into the endoderm; activation of PI3K is mainly thought to be regulated by IGF2 and bFGF2. Picture adapted from (136).

1.2.7 Passaging techniques of hiPSC

It is, for obvious reasons, important to prevent hiPSC from spontaneous differentiation in culture. Depending on the cell line and derivation, the doubling time of these cells can differ, but in general they have a doubling time of 35-40 hours, and need to be passaged with a 3-4 days interval. The cloning capacity of hiPCS is poor in standard culture conditions. The cell-cell junctions between hiPSC are similar to those found in epithelial cells, consisting of tight junctions, asymmetric distribution of organelles and cytoskeletal components (137). Binding of cells to the matrices is necessary for their survival, which involves β1 and α integrins

(138). If cells are not tightly controlled, they in undergo a process termed anoikis, a programmed cell death occurring upon detachment of cells from their ECM causing disorganization (139). Various passaging techniques have been established in order to propagate hiPSC in a simple and effective way. Mechanical passaging involves scalpels or cell scrapers to "cut and paste" small colonies from one plate to another (134). Enzymatic passaging uses enzymatic dissociation reagents to obtain single cells, or enzyme-free dissociation, i.e. EDTA-based approaches (140). As mechanical passaging is time-consuming, laborious and does not yield high numbers of cells, single cell dissociation techniques are favored. Enzymatic dissociation of hiPSC disrupts the cell-cell interactions and the actin-myosin contraction (141), causing cell death of especially single cells (142). Once the cells are removed from their ECM environment, and cell-cell contact are lost, the balance of actin-myosin contractions is disrupted allowing free contractility. This leads to constitutive activation of Rho-associated coiled-coil kinase (ROCK). Active ROCK leads to plasma membrane blebbing and eventually cell death (143). The use of Rho-associated coiled-coil kinase (ROCK) inhibitor (ROCKi, Y-27632) has been used for single cell passaging and can save single passages cells up to 27% (142). Two hypothesis exist for how ROCKi functions:

1. When ROCKi is used, the blebbing of the plasma membrane is completely stopped and anoikis is inhibited (143).
2. Using ROCKi during cell dissociation prevents the cells from sensing their surrounding environment, and allowing them to make important cell-cell and matrix connections needed for survival (144).

ROCKi is widely used in human PSC cultures to allow for survival and expansion of large number of pluripotent cells. Cell number and pluripotency is of critical value for various analyses *in vitro*. It is required for studies involving cell based therapy approaches, but also to avoid clonal selection of differentiated cells carrying abnormal features during long culture periods.

1.2.8 Differentiation of hiPSC

hiPSC have the ability to form any cell type in the body, and ironically, this ability to form any cell type in the body is also one of the difficult tasks to control for *in vitro*. The fate of hiPSC is depended on the chemical and physical signals from its microenvironment, either signaling molecules or cell matrix surfaces. Depending on which cell fate hiPSC commits to, the lineage specific genes will be turned on, and the core pluripotency factors will be silenced. Differentiating cells were described as marbles rolling down a hill with different developmental potentials at different valleys, and as the cells reached the bottom of the hill – their fully differentiated state was achieved. One key in differentiation is the change in epigenetic landscapes, where PSC are distinctly different from lineage-committed cells (145). DNA methylation patterns have shown that amounts of methylated regions are significantly increased in lineage-committed cells as compared to those seen in PSC (146-148). DNA methylation in differentiation also silences the core pluripotency factors such as Oct4 and Nanog (149), and retroviral factors in PSC are targets for *de novo* methylation by specific Dnmt's (150). Different hiPSC lines can have different differentiation potentials depending on their original cell type (151). In terms of differentiation, caution has to be taken when deriving a certain cell type *in vitro*. This because the epigenetic and also transcriptional machinery is still to immature for complete differentiation of a desired cell type and can lead to a sustained epigenetic memory in the derived cell types (151).

1.2.9 Epigenetic memory in hiPSC

Different tissues in have various degrees of sensitivity to reprogramming. The most prominent cell type in the epidermis, the keratinocytes, are more susceptible to reprogramming than e.g. fibroblasts (152). iPSC from adult tail-tip fibroblasts are more prone to form teratomas than iPSC from embryonic fibroblasts (152, 153) underlining that iPSC derived from different tissues can display differences in function and molecular patterns. DNA methylation in transcription factor based reprogramming occurs over days and weeks in iPSC derivation (154). Also, when iPSC were compared regarding their DNA methylation state and cell tissue origin, it was found that blood-derived iPSC could yield more hematopoietic colonies than fibroblast-derived iPSC. However, the fibroblast-derived cells produced more osteogenic colonies, deposited more calcium and expressed more osteoblastic gene markers (155). The DNA methylation state of these cells showed there were differences between the blood derived iPSC and the fibroblast iPSC. As an example, 11 of the hematopoietic loci were hypomethylated in the fibroblast iPSC (155). Comparative analyses of human ESC and iPSC with their initial somatic cell origin showed a similar methylation pattern of 79.5% at CpG sites, suggesting that only 20% undergo methylation modification. These were regions mostly associated with genes of transcription (156, 157). The reprogramming of somatic cells to iPSC is followed by different DNA methylation patterns and also different amounts of methylation, depending on cell type of origin. It is worth mentioning that the majority of different methylation regions arise from de novo methylation, and a smaller part is associated with epigenetic memory. The imprinted genes in early iPSC have prevented murine iPSC to form chimeras through tetraploid complementation, and failed to support development and a fully iPSC derived animal (158). Surprisingly, stem cells derived from somatic nuclear cell transfer underwent more complete reprogramming than iPSC. Thus, the nuclear transfer ESC showed threefold less aberrant DNA methylation pattern compared to iPSC (159). The epigenetic phenomena in iPSC need attention and need to be considered when iPSC are used for disease modeling or regenerative medicine. As incomplete reprogramming be expressed as re-activation of genes related to stem cell maintenance but not pluripotency, and also incomplete repression of the lineage specific transcription factors.

1.2.10 Therapeutical potential of hiPSC

Human iPSC are patient specific stem cell that are well suited for modeling human diseases *in vitro*, as they carry the same genetic setup as the somatic cells they were derived from. These cells can be differentiated into any cell type in the body and can present the same phenotypes as those seen *in vivo*. Primary cells from patients are usually available in small quantities, whereas hiPSC are present in large numbers and can also be engineered to form any cell type of interest. One of the first iPSC-based models for disease was designed to study spinal muscular atrophy (160). Since then, many more iPSC models have been used, e.g. for hepatic disorders (75), hematopoietic disorders (161), Huntington's disease (162) cardiac disorders such as the long QT syndrome (163), Parkinson's (164), and Alzheimer's diseases (165).

Furthermore the disease phenotypes can be corrected with gene editing approaches. Here, a mutation in e.g. osteoblasts that were derived from iPSC cells from a patient with a congenital bone malformation syndrome can be switched and the wild-type phenotype "reestablished" (166). Such techniques could be of use for future cell based therapy approaches, where gene-corrected iPSC could be transplanted back into a patient and rescue a mutation (167). In this context, it should be stressed that much care has be taken to correct

the mutation without introducing new and potentially fatal mutations. This particular problem could be dealt with by means of fine-tuning the technique through whole genome sequencing. This would sort out screens of corrected iPSC before they are transplanted back into the patient. This approach has been used *in vivo.* Thus*,* a corrected gene from iPSCs derived from patients with β-thalassemia, an inherited autosomal recessive blood disorder characterized by abnormal formation of hemoglobin, was transplanted into mice. This lead to a normalization of hemoglobin production in red blood cells in the mice (167). Furthermore, the pro-apoptotic phenotype of motor neurons from patients with amyotrophic lateral sclerosis (ALS), could be rescued after a similar genetic correction of in this case the SOD1 gene had been performed (168).

It must be noted, that currently, disease models fashioned by hiPSC do not reflect the *in vivo* environment to the extent that animal models can. However, there are reasons for optimism regarding rapid improvements of iPSC-based systems both with respect to disease modeling and use in the clinics.

1.3 **EPIGENETICS**

The term epigenetics can be defined as heritable changes in gene expression and phenotype without changing the underlying DNA sequence. Epigenetic research has become essential for the understanding of how genes are regulated and how expression programs are set for different cells types, and further how these are changed especially in PSC and during disease. In broad terms, epigenetic alterations, including DNA methylation, histone modifications, and nucleosome remodeling are all involved in regulating the accessibility of DNA on a heritable level. This influences the accessibility of transcription factors, and histone remodeling complexes to DNA, thereby altering the genomic structures and influencing gene expression.

In every cell of the body, the approximately 2 meter long DNA strand is contained within every nucleus, and is influencing every everything in cells and tissues throughout any organism. This long strand of DNA is in need of a smart packaging system in order to fit into the small nucleus, which is approximately 5-20µm. The small basic proteins called histones provide the solution to this. These consist of an octamer of H2, H2a, H3 and H4. The histones neutralize the negative charge of DNA, making it extremely condensed. There are 147 base pairs of DNA wrapped around each histone, making up the so-called chromatin. Histones are linked with a protein called linker histone, H1 (Figure 5). However, the very condensed DNA still needs to be available for transcription, replication, and repair. The unpacking of DNA is controlled by DNA methylation, histone modifications, RNA-associated modifications, and histone variants.

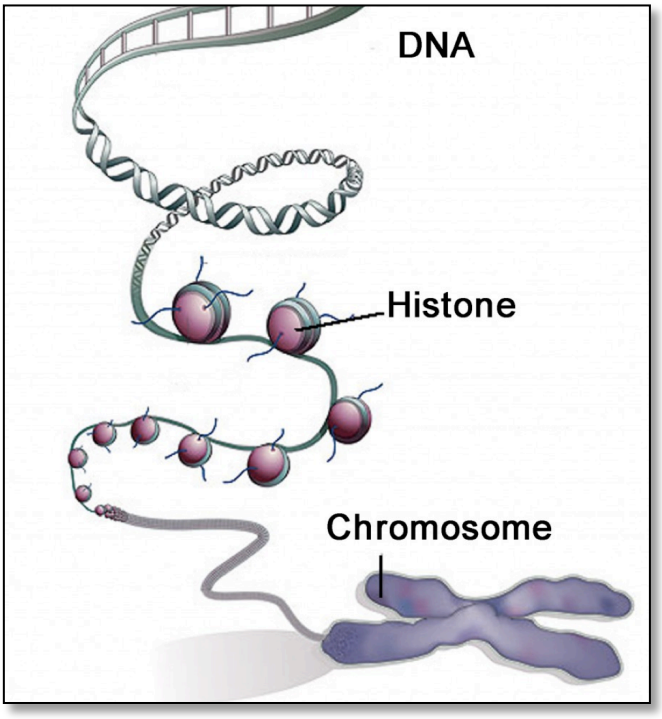

Figure 5. The chromosome, histone, and DNA.

Around each histone 147 base pairs of DNA are tightly wrapped, making the long DNA strand very condensed. The histones consist of an octamer of H2, H2a, H3, and H4. Epigenetic control of genes occurs mainly through DNA methylation, or modifications of the histones through their protruding N-terminals tails. Picture adapted from (169).

1.3.1 DNA methylation

DNA methylation was discovered already when DNA was identified as the genetic material, but it was only in the 1980's when it was first demonstrated that it had a role in gene regulation and cell differentiation. DNA methylation is a more stable and inheritable epigenetic pattern when compared to chromatin modifications, and can last over generations (170). Almost in every cell, most of the DNA is methylated, but CpG islands in close proximity to promoters of housekeeping genes are an exception. Most of DNA methylation, approximately 80%, occurs at these so-called CpG islands (171, 172). These regions are suggested to have a functional importance in evolution, especially those associated with promoters, and they are also conserved between mice and humans (173). CpG islands are strands of DNA around 1000 base pairs long and have a higher CpG density than the rest of the genome, and are frequently not methylated. Approximately 70% of gene promoters are found within CpG island (171, 172). This implies that these regions have a functional role in evolution and are important in gene expression. Methylation of CpG islands can result in stable silencing of gene expression, impaired transcription factor binding, and also recruitment of repressive methyl-binding proteins. DNA methylation is essential for silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and X chromosome inactivation. In contrast to the regions in the genome with high density of CpG, there are other regions, which contain few CpG sites and are largely methylated, these are called CpG "shores", and located approximately 2kB away from CpG islands. DNA methylation can directly silence genes located within these shores and further contribute to methylation patterns seen in different tissues (174). Most genes controlling the expression of tissue-specific genes do not lie within CpG islands (175), and genes within non-CpG island promoters have been reported to be methylated in normal tissue, which suggests for a contribution of CpG shores in tissue expression patterns.

1.3.2 Regulation of DNA methylation

The enzymes responsible for DNA methylation are called DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b). They transfer methyl groups to cytosine residues (176, 177). Dnmt1 is the maintenance methyltransferase, and essential during development. Knockout of Dnmt1 in mice results in embryonic lethality and loss of genomic imprinting (176). Dnmt1 is active during DNA replication and is responsible for copying DNA methylation pattern from the parental strand to the newly synthesized daughter strand, but also for repair of DNA methylation. Dnmt3a and b are in charge of *de novo* DNA methylation and highly active in PSC, where they methylate unmodified DNA. Removal of these enzymes from PSC leads to inhibition of *in vitro* differentiation (177). Dnmt3a has been suggested to be important in early development, as knockout of Dnmt3a is embryonically lethal. Dnmt3b has been proposed to be necessary for normal tissue and cellular differentiation, since Dnmt3b knockout mice survive for 4 weeks after birth (177).

DNA methylation operates by several mechanisms.

1. DNA methylation of promoters sites can impair transcription factor binding to the gene (178).
2. Methylated DNA can be bound by proteins called MBDs. MBDs may then, in turn, recruit other proteins that modify histones and silence gene expression. This leads to changes in chromatin structure (179).

DNA methylation provides long-term silencing crucial for an organism for processes as those mentioned above. However, DNA methylation also undergoes dynamic changes leading to distinct cell types and tissues, both through the regulation of methylation, but also through a process called demethylation. Genes needed during a later time-point in development need to be repressed early in life, and become active at the right time with the right signals. This is a delicate regulation and is highly controlled. Aberrant DNA methylation has been associated with cancer and other imprinting related disorders (180). Inactive DNA demethylation is achieved through inhibition of Dnmt1 (154) or through active demethylation by the enzymatic removal of a methyl group from 5 methylcytosine (181, 182). TET family of enzymes consists of TET1, 2 and 3 with a core catalytic domain. TET enzymes recognize modified C bases in DNA and through catalyzation of oxidative decarboxylation it generates an enzyme, which converts 5mC to 5hmC (183). Base excision repairs have also been suggested in demethylation, where the enzyme thymine DNA glycosylase (TDA) possesses a mechanism, which excises 5mC directly. TDA is required for embryonic development and TDA null embryos are epigenetically abnormal, with decreased developmental transcription factors such as the HOX genes (157, 184).

1.3.3 DNA methylation in pluripotency

During development stem cells differentiate into various cell and tissue specific fates. This process is established through a complex transcriptional and epigenetic network. A very traditional way for stem cell commitment is that lineage differentiation has been directional and irreversible. Conrad Waddington, who developed the classical concept of the epigenetic landscape, illustrated this with his famous model. The epigenetic landscape was illustrated by a marble rolling down a hill towards terminal differentiation, with groves and different slopes representing different instructions for the marble to reach its final fate. With the discovery of iPSC, where the mature cell is reprogrammed into pluripotency, the landscape lost its unidirectional fate Figure 6.

DNA methylation combined with chromatin modifications in PSC regulates gene expression and is also responsible for tightly regulating transcription upon differentiation. Methylated CpG islands in PSC are generally present at promoters of repressed genes. Unmethylated genes are associated with housekeeping genes and pluripotency genes, such as Oct4, Nanog, and Sox2, allowing for high levels of these transcription factors (185, 186). Methylation levels of CpG islands in PSC are less than 20%, suggesting that most of the genome in PSC is unmethylated (148). In these highly unmethylated regions of CpG at promoters, housekeeping genes for pluripotency are enriched with H3K4me3 (127). Moreover, iPSC inherit DNA methylation states as an epigenetic memory from the parental cell origin, suggesting for a memory influence from the parental cell origin (156). This epigenetic memory can include persistent DNA hypermethylation of genes necessary for pluripotency. As DNA methylation is not a static state, cell lines that have lost their DNA methylation by using the drug 5-aza-cytidine, has shown to go from a partially reprogrammed state into full pluripotency. This has been explained by the inhibition of Dnmt1 (154).

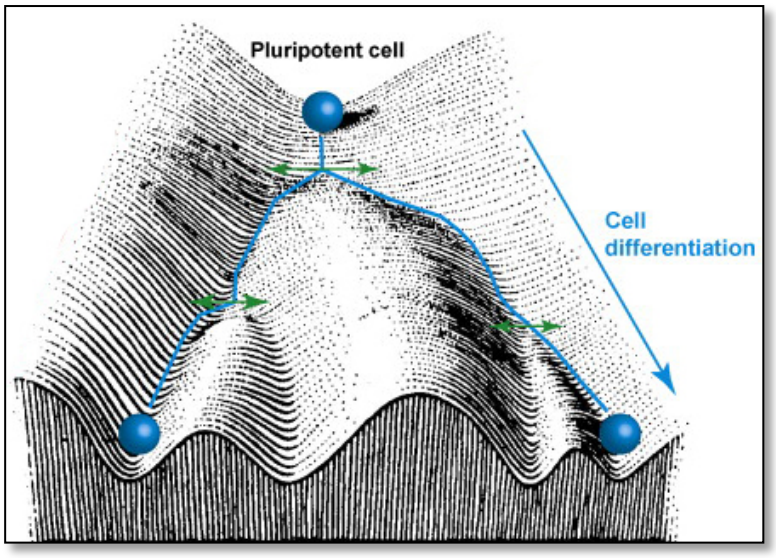

Figure 6. Conrad Waddington's epigenetic landscape.

Conrad Waddington's epigenetic landscape shows how a cell becomes more and more determined during development and differentiation. This is illustrated by a marble rolling down a hill. The green arrows represent cells that are partially or fully differentiated. However, with iPSC technology today the reprogramming factors have the ability to drive the marble back up to a pluripotent stage. Picture adapted from (187).

1.3.4 Chromatin modification

Walther Flemming's interest in cell division lead him to the first discovery of chromatin in 1882. He developed a new histological staining suspension called "Flemming's solution", which enabled him to visualize fibrous structures in the nucleus. He subsequently named these structures "chromatin" (stainable material) (188). Almost half a century later, in 1928, Emil Heitz also laid one of the cornerstones in modern epigenetics. Using an in situ method, he established the terms "euchromatin" (genetically active) and heterochromatin (genetically silent). He described euchromatin as a more sparsely stained chromatin, and heterochromatin as much more compact and densely stained. Posttranslational modifications of the N-terminal of core histone proteins modulate chromatin structure and are crucial for maintenance of gene regulation (189) Figure 7. Their protruding histone tails are subjected to various post-translational modifications, including acetylation, methylation, phosphorylation, and ubiquitylation. Histones are basic and by adding acetyl groups the balance is shifted and the histones modify the accessibility of DNA. The most studied modification is methylation, where histone methyltransferases (HMT's) add methyl groups, and histone demethylases (HDM's) removes methyl groups. In acetylation, histone acetyltransferases (HAT's) acetyl groups are added and can be removed by histone deacetylases (HDAC's). Depending on the site of modification and the modification itself, the gene can be either active or repressive and then further influenced by recruited modifying enzymes. Methylation can act differently depending on which residue it acts on (170). As examples, histone 3 lysine 4 thri-methylation (H3K4me3) and acetylation are associated with transcriptional activity, in which the mark works as a signal for the recruitment of RNA polymerase and promotes activation. Histone 3 lysine 27 thri-methylation (H3K27me3) is associated with transcriptional repression, by recruiting silencing factors (170).

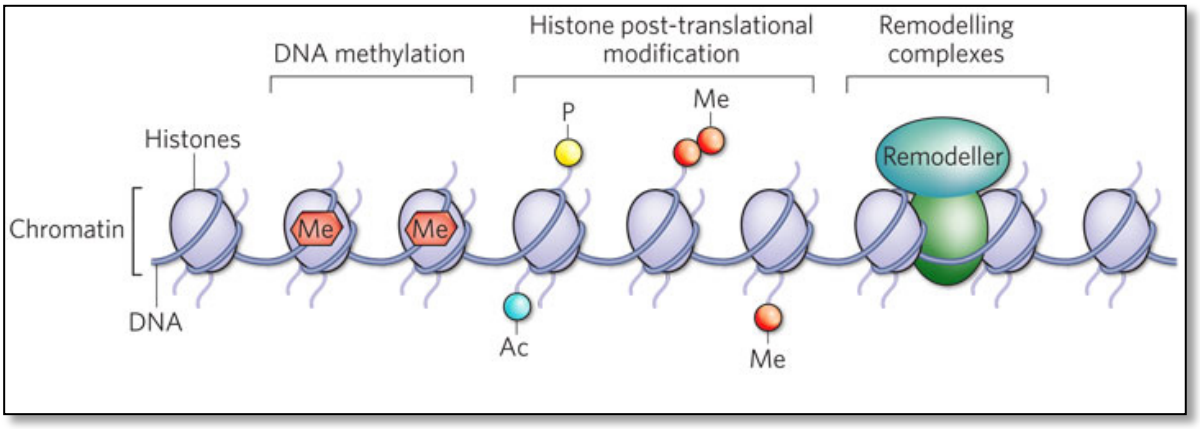

Figure 7. Factors involved in chromatin modifications and regulation.

The protruding histone tails are subjected to various post-translational modifications, including acetylation, methylation, phosphorylation, and ubiquitylation. Chromatin structure is also affected by DNA methylation, and remodeling complexes. These are all crucial to development and play an important role in tissue and organ determination. Ac – acetyl, Me – methyl, P – phosphate. Picture is adapted from (190).

1.3.5 Chromatin in pluripotency

Chromatin in PSC is believed to generally have a more open state as compared to mature somatic cells. Chromatin has shown to be less dense in PSC, and the ratio between euchromatin and heterochromatin is much higher than in somatic cells and/or cells undergoing differentiation. The genome of mouse ESC has been shown to be transcriptionally hyperactive with many chromatin remodelers, as compared to heterochromatin in differentiated cells (191). This has also been shown *in vivo* in the mouse, where ICM in the blastocyst shares the same chromatin pattern as in PSC. ICM has a lower number of dense clusters of heteropacked chromatin, and is composed more of euchromatin where the chromatin is more accessible in comparison to lineage-committed cells (192). Furthermore, data analysis from ChIP-seq has shown that the epigenomic landscape of hESC of chromatin markers H3K9me3 and H3K27me3 increased during differentiation (193), where both these methylation marks are associated with heterochromatin. This increase of histone repressive marks is a way to silence the pluripotency markers, and allowing cell specific markers to be expressed. Interestingly, the marker H3K9ac which is associated with transcriptional activation has a genome-wide reduction in differentiated cells (194).

1.3.6 Epigenetic regulation of pluripotency in PSC

Regions of the genome in PSC are “bivalently” marked and are ready for signals to either activate the region of interest or silence the region. The transcriptional repression-associated marker H3K27me3 and the activation-associated transcription marker H3K4me3 are both found at the same sites in the genome of PSC, and are catalyzed by polycomb-group proteins and thrithorax, respectively (195, 196). They are believed to regulate PSC by silencing developmentally regulated genes, while simultaneously keeping them ready for various lineage-specific genes through loss of H3K27me3 (196). There is generally an increase in heterochromatin during PSC differentiation, which may directly lead to silencing of the regulators involved in maintaining pluripotency in PSC. The proteins involved in chromatin remodeling are divided into four different families: SWI/SNF, CHD, ISWI, and INO80, and

all of them use ATP hydrolysis to change the DNA-histone complex. The remodeling complexes have a catalytical region with an ATPase domain required for the remodeling of the DNA-histone complex, a unit for the recognition of chromatin and the nucleosome, and a recognition site for interaction with other proteins necessary for remodeling or transcription factors important in gene regulation (197).

Thri-methylation of lysine 27 on histone 3 (H3K27me3) is associated with negative regulation of gene transcription (198) and a more compacted chromatin. In undifferentiated cells H3K27me3 occupancy in the genome is 4-fold higher compared to differentiated cells (193). H3K27me3 is regulated by PcG, which is recruited by Jarid2. Inhibition of Jarid2 leads to great loss of PcG binding and subsequently to a decreased level of H3K27me3 (199). Jarid2 is both responsible for targeting H3K27me3 and fine-tuning, by recruiting PcG proteins such as polycomb repressive complex (PRC2), but also inhibiting the methyltransferases activity (199). Methylation is directed by the enzymatic chromodomain portion of PRC2 called, EZH2, which recognizes H3K27me3 (200). Further PRC2 occupies a special set of genes involved in development, organogenesis, regulation of transcription, and neurogenesis (201). These genes need to be repressed in order to maintain pluripotency in ESC, but can be activated upon differentiation signals. Thus PRC2 is thought to function as both a repressor and regulator of both differentiation and pluripotency.

Tri-methylation of lysine 4 on histone 3 (H3K4me3) is strongly associated with transcriptional activity, where it has been shown to occupy 75% human gene promoters in PSC and has a strong correlation with gene transcription (202). H3K4me3 is regulated by the thrithorax group (TrxG) and recognized by a bromodomain and PHD containing transcription factor of the NURF protein complex (202, 203). By recruiting methyl groups to lysine 4 on histone 3 and further recruiting nucleosome-modeling enzymes it leads to transcriptional activity. The HMT responsible for the methylation are regulated by a SET domain, and is under further control by specific enzymes (204).

Tri-methylation of lysine 9 on histone 3 (H3K9me3) is another transcriptional repressive-associated histone mark important in PSC is H3K9me3. It has been shown to be increased with differentiation and is expressed at low levels in undifferentiated cells. The low levels seen in undifferentiated cells are maintained by a positive feedback loop of the Oct4 promoter by HDMT Jmjd2c. Jmjd2c is responsible for demethylating H3K9 at the promoter of Oct4 and thus leading to activation (205). Depletion of Jmjd2c leads to increase of H3K9me3 and differentiation, as seen by changes in cell morphology and decreased alkaline staining (205). The high levels of H3K9me3 in differentiated PSC are maintained by a SET-containing HMT, G9a, which also silences Oct4 upon differentiation (149). G9a binds to Oct4 promoter and leads to methylation of H3K9, followed by Dnmt's to further stabilize the repressive state at the promoter site. Adding to the evidence of the importance of H3K9me3 in PSC, analyses of ChIP-seq data have shown that in undifferentiated cells the genome occupancy of ESC is around 4%, but increases 3-fold upon differentiation (193).

1.3.7 Epigenetic crosstalk in pluripotency

Chromatin modifications and DNA methylation do not work alone; there is a constant crosstalk where one can influence the other. This is important, as both these posttranslational modifications are important for maintaining pluripotency, but also for the mature differentiated state of a somatic cell in the body. DNA methylation has a direct role in regulating the chromatin structure. Methylated CpG islands are mainly observed at promoters of repressed genes in PSC, and they are also correlated with low levels of H3K4me3 (206).

The presence of the active histone modification marker H3K4me3 hinders the binding of Dnmt3a and Dnmt3b to H3 histone tails, thus preventing methylation (207). Furthermore, methylated CpG islands are usually correlated with high levels of unmethylated H3K4 and H3K9me3, and can help keep the CpG islands hypomethylated (127). It has been proposed that increased levels of H3K9me3 and H3K27me3 are associated with decreased levels of DNA methylation in differentiated cells. Hence, in differentiated cells H3K9me3 contains almost half as much CpG methylation as in hESC, and the levels for CpG methylation at H3K27me3 regions are also much lower in differentiated cells compared to hESC (193). In addition, HMTs can directly interact with other modifying enzymes of histones, by adding or removing histone marks, and thus create a repressive or active gene (208). One example is HDACs, that interact with HAT and force the removal of acetyl groups from lysine residues, which makes the DNA tightly packed (209). Furthermore, in hESC H3K4me3 was found to be inversely correlated with methylation when compared to differentiated cells, and H3K27me3 was associated with hypomethylation of DNA at promoter sites (193).

2 AIMS OF THESIS

The overall aim of this thesis is to enhance the understanding of stem cell differentiation into the osteoblastic/mineralizing lineage. This is made in several steps, which span from studies of i) basic biology of cellular calcification ii) stem cell culture techniques and iii) and using next generation sequencing to elucidate the molecular pathways involved in skeletal malformations in iPSC derived from a family with a PIG-T mutation.

SPECIFIC AIMS

❖ STUDY I

The aim of study I was to investigate the distribution of OSAD in the developing mouse tooth and compare OSAD with other known markers important for minerlization. Further, we wanted to use an *in vitro* model system with rat dental pulp cells exposed to mineralizing factors to study the role of OSAD in mineralization.

❖ STUDY II

The aim of study II was to gain insights into the biology of stem cells for subsequent applications in the field of stem cell/regeneration of of bone. Specifically, the objective was to investigate how the epigenetic status of ESC was affected by passaging techniques in the laboratory.

❖ STUDY III

The aim of study III was to elucidate transcriptional gene expression changes and epigenetic alterations that might be responsible for skeletal malformations in patients with a mutation in the PIG-T gene.

3 MATERIAL AND METHODS

3.1 **Ethics statement (Studies I-III)**

3.1.1 Study I

All animal experiments were performed in accordance with the current legislation in Sweden and after approval from the Karolinska Institute Ethical Research Board (S52-08, S53-08, S179-06).

3.1.2 Study II

For all hESC work, ethical approval from the Ethics Committee of Karolinska Institute has been obtained (Dnr 454/02). Informed consent from both parents has been given for embryo donations after receiving both oral and written information.

3.1.3 Study III

All hiPSC cell lines were obtained under signed informed consent, and ethical approval was given from ”Regionala etikprövningsnämnden i Stockholm” (Dnr 2012/208-31/3).

3.1.4 Tissue sectioning and light microscope immunohistochemistry (Study I)

In order to investigate the distribution of SLRP’s in dentin development, with specific interest in OSAD, tissue from mice were collected from different time points in development (embryonic (E) days E15, E17, newborn (NB), day 5 (d5) and adult (> 3 months)). The tissues were fixed in PFA, and decalcified in EDTA to enable sectioning. Following dehydration and embedding in paraffin, sections for BGN, FMD and DCN were treated with chondroitinase ABC as the antibodies against these SLRPs only recognize the protein core, which lack GAG chains (chondroitinase removes the GAG chains) (210, 211). Sections were stained with horseradish-peroxidase (HRP)-conjugated antibodies against OSAD, FMD, BGN and DCN. Before incubation with antibodies, tissues were treated with H_2O_2 for removal of endogenous peroxidase activity. Bound antibodies were detected using the DAB substrate, which generates a brownish reaction product where the protein of interest is expressed. Sections were counterstained with hematoxylin and eosin, which stains cells nuclei in blue and the cytoplasm and the surrounding extracellular matrix in pink. Staining intensity was evaluated, scored, and plotted in a table.

3.1.5 Ultrastructural localization (Study I)

Mandibles from the time points listed above were dissected out, fixed and embedded as previously described (212). Ultrathin sections were cut and placed on formvar coated one-hole nickel grids. They were incubated with OSAD antibody and the primary antibody was detected with rabbit anti-goat antibody conjugated with 10 nm gold particles, followed by analysis in a transmission electron microscope. Ten random images from each dental compartment were taken (cell layer, predentin, dentin and enamel). The density of OSAD gold labeled particles was calculated in each image and the result expressed as mean number of gold particles per μm^2 ($Au/\mu m^2$).

3.1.6 In vitro mineralization (Study I and III)

Using *in vitro* cell culture systems, the developmental expression of tissue-specific genes and phenotypes can be studied in an environment resembling the *in vivo* milieu. In study I, rat dental pulp cells (rDPC) were used to model biomineralization. Pulps were dissected out from rat third molars and digested with collagenase. Cells were isolated and placed in

appropriate cell media. A mineralizing phenotype was induced by seeding the rDPC at 28500 cells/cm^2 in standard mineralizing media (α-MEM, 10% heat-inactivated FBS, 1% P/S, 50μg/ml ascorbic acid phosphate and 10mM β-glycerophosphate). For study III we wanted to stimulate hiPSC of patients into an osteogenic lineage and compare it with healthy hiPSC. We used standard osteogenic media as above, but as the optimal use of dexamethasone is still not settled we also used a concentration of 100nM dexamethasone during the first week (31). Control experiments were run in parallel without the addition of osteogenic supplement. The experiments were performed for 21 days and were repeated on three separate occasions.

3.1.7 Assessment of the mineralizing phenotype (Study I and III)

Alizarin Red S staining, which detects calcium deposition, was used as an indicator of mineralization. Cells were stained with 40 mM Alizarin Red S (pH 4.2). In study I calcium content was quantified by measuring the amount of Alizarin Red S staining, which was bound to the mineralizing nodules. Thus, after staining cultures was briefly washed and extracted with cetylpyridinium chloride. The concentration of the dye was determined by absorbance at 540 nm. Calculation of staining was based on the Alizarin red S and a known standard.

3.1.8 RNA analyses (Study I-III)

Throughout study I-III we have used ribonucleic acid (RNA) to measure gene expression in our cell culture systems. RNA is responsible for coding, regulating, and expressing the genes, which are encoded by our DNA. Conventional polymerase chain reaction (PCR), where the product is measured at the end, allows for the analysis of mRNA. mRNA is the machinery, which transfers the information necessary for the building of proteins, and is transcribed into stable complementary DNA (cDNA) and amplified to a level of detection by PCR. q-PCR measures the product in real time as compared to traditional PCR. We have used Taqman and SYBR green probes throughout the studies. Taqman probes are labeled with a fluorescent reporter at the 5' end and a quencher in the 3' end. This allows detection only when there is a specific hybridization of cDNA with probe. Further, when it is bound and the cycle is repeated, it will break up the probe and release the reporter dye. As this is repeated in every cycle, the reporter signal will be directly proportional to the amplified product. SYBR green probes were designed with the program "Primer Express". These are in difference to other probes, such as Taqman probes, as they will bind to all double stranded DNA products and might have a higher risk of nonspecific binding and primer dimer.

3.1.9 RNA extraction and quantitative real-time polymerase chain reaction (q-RT-PCR) (Study I-III)

Total RNA was extracted from all cells throughout all studies using a commercial RNA kit from Qiagen. RNA concentration was quantified with a spectrophotometer at 260/280 nm using Qubit. cDNA was made using a high capacity reverse transcriptase kit and samples were then used for q-PCR analyses. GAPDH was used as a housekeeping gene for studies I and III, and 18S was used for study II.

3.1.10 Derivation of human embryonic stem cells (study II)

The four hESC used in study II were all previously derived and characterized. Briefly, supernumerary embryos were donated to the Fertility Unit at Karolinska Institutet. Fertilization of oocytes and embryo were performed by embryologists and then scored depending on their quality. Embryos with highest scores were always given to the couples,

and only those embryos, which could not be used for infertility treatment, were used in hESC derivation. The inner cell mass was isolated and then plated onto appropriate feeder plates. For further details of the derivation of hESC, see (213, 214).

3.1.11 Derivation of hiPSC – skin fibroblast cell culture (Study III)

In theory, any somatic cell type can be used for the derivation of iPSC. Here we have used skin biopsies from 4 patients with PIG-T mutation (1 heterozygote healthy, 3 homozygous sick, and one from a healthy non-related donor). The explants of human skin were minced, digested, and placed in a culture dish with 0.1% gelatin in appropriate media. They were kept at 37^{o}C in 5% CO^2 where media were changed every other day. The fibroblasts were used at first passage for reprogramming, freezing or further passaging.

We reprogrammed the fibroblasts in fibroblast media for 2 days using the non-integrating CytoTune^{TM} – Sendai viral vector kit (Moi 3). Cell morphology changes could be seen at day 7-10 and cells were then replated onto on mitotically inactivated (irradiation 40 Gy) human foreskin fibroblasts in basic iPSC media. At day 21-28 of differentiation iPS like colonies were manually picked and individually expanded. Several clones where propagated and characterized from each sample. One clone from each of the 5 lines was transferred from hFF to Matrigel®, a basement membrane extracted from Engelbreth-Holm-Swarm tumor.

3.1.12 Cell culture medium (Study II and III)

Human ESC and iPSC need to be maintained in undifferentiated states, and thus strict culture conditions are crucial. For both hESC and iPSC we used a standardized medium containing KnockOut Dulbecco's modified Eagle's medium, supplemented with 20% KnockOut Serum Replacement, 2 mM glutamax, penicillin streptomycin 0.5%, 1% nonessential amino acids, 0.5 mM 2- β_2-mercaptoethanol, and 8 ng/mL of basic fibroblast growth factor. bFGF2 is important for the cells to remain pluripotency and is thus essential for culture medium. Commercially available medium such as mTeSR^{TM}1 and NutriStem® hESC XF was used when the cells were on feeder free matrix (Matrigel®). For study II and III, mTeSR^{TM}1 and NutriStem® hESC XF were used, respectively.

3.1.13 Cell matrices (Study II and III)

Commercially available post-natal hFF was used in Study II and III to minimize xeno-components in culture conditions (215). hFF were grown in fibroblast medium consisting of Iscove's modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS), and 0.5 % penicillin streptomycin. When confluent, the cells were irradiated (40 Gy) and plated down as cell matrix. The day after plating, FBS in the medium was replaced with SR. 3 days after plating, the medium was replaced with hESC and iPSC media as mentioned above, and the matrix was ready for use. Cells from both studies were transferred onto Matrigel® coated plates for experiments.

3.1.14 Passaging techniques

Pluripotent cells can be expanded for indefinite periods of time and still maintain their pluripotent state. This requires continuous passaging using different techniques involving cell scrapers, different enzymatical solutions or surgical scalpels were the cells are manually "cut". Once dissociated the cells are transferred to freshly made matrix plates.

3.1.15 Passaging of hESC (Study II)

All four hESC were transferred from hFF plates onto Matrigel®. They were passaged in three different ways for a total of three passages before samples were collected for analysis.

i) Mechanical, were a surgical scalpel was used to "cut" the colonies (based on their morphology) into small pieces (111).
ii) Enzymatical + ROCKi, enzymatical passaging using TrypLE select with the addition of ROCKi. Briefly, the cells were preincubated with 10 μM ROCKi 1hour in 37°C in 5% CO^2 prior to passaging with TrypLE select. Cells were centrifuged, resuspended in pre-warmed media supplemented with 10 μM ROCKi and plated onto freshly coated Matrigel® plates.
iii) Enzymatical – ROCKi, enzymatical passing using TrypLE select. Procedure was as above without the addition of 10 μM ROCKi.

3.1.16 Passaging of hiPSC (Study III)

All hiPSC lines were transferred mechanically from hFF plates to Matrigel® coated plates. They were kept for three passages until they were collected for analysis (three biological replicates from each individual cell line). Cells were dissociated using TrypLE select, briefly; media were removed and cells were incubated with TrypLE select for 3 minutes in 37°C in 5% CO^2. The cells were collected and centrifuged for 1.300 rpm for 3 minutes), re-suspended in pre-warm hiPSC media and plated accordingly.

3.1.17 Immunocytochemistry (Study II)

Immunocytochemistry is a widely used method to detect the distribution and presence of specific proteins of interest. The major difference between IHC used in study I and ICC is that the former has most of its extracellular architecture and tissues intact, while ICC has most of its extracellular matrix removed. All hESC were fixed in PFA, cells were washed and permeabilized using Triton X-100. For stress fiber markers, the cells were incubated with blocking solution, and then incubated with the primary antibodies for the pluripotency marker NANOG, and the stress fiber marker phallodin (binds to F-actin). Nuclei were stained using DAPI. All lines were also stained for the nuclear markers OCT-4, NANOG, and SOX2. Signals were detected using a fluorescent microscope and images were captured using a CCD camera.

3.1.18 Flow cytometry (Study II)

Flow cytometry is a laser-based method in which cells can be monitored, sorted, and analyzed in real time. Cells travel in a stream of fluid, which passes a detection system that allows simultaneous analysis of the characteristics of the sample of cells. In addition to the antibodies used for ICC, we investigated a general marker of pluripotent cells; TRA-2-54. This is a marker for alkaline phosphatase that stains pluripotent stem cells (216, 217). A total of 50 000 cells from two hESC lines were used for analysis. Cells were harvested and trypsinized to obtain single cells. They were diluted in 5% FBS in PBS, and the antibody against TRA-2-54 was added according to the manufacturer's protocol. Following incubation with primary antibody, cells were washed and a secondary antibody was added for the flow cytometer to detect a signal from individual cells. The flow cytometer records forward scatter (cell size) and side scatter (cell granularity), as well as fluorescent intensity, which is directly related to the expression of the targeted protein.

3.1.19 Chromatin immunoprecipitation (ChIP)

ChIP technology is a method used in the lab for the identification of and localization of histone modifications. The cells of interest are cross-linked with formaldehyde to allow all proteins to bind to DNA. Cells are then lysed to fragments ranging from 500-1000 base pairs (2-3 nucleosomes) using an enzyme or a sonication based method.

i. Enzyme based method: This is more gentle to cells and keeps the epitopes intact, thus inrcreasing the interaction of samples with the antibody. Enzyme based methods can also give increased detection of DNA loci, and requires less material. However, given these benefits, the enzymes can interact with downstream reactions and with lower resolution as a result (resolution here is referred to as the separation of fragments to obtain the DNA-chromatin complex). This is because the enzyme does not possess enough recognition sites in order to yield as high resolution as other methods can.
ii. Sonication based method: This is not an enzyme based method, instead a sonicator or a nebulizor is used. The fragmentation is random and produces fragments which give different resolutions, depending on short or long sonication times. A short sonication will result in a high percentage of chromatin but low resolution, whereas a long sonication results in low chromatin percentage but high resolution.

All fragments from sonication are controlled with gel electrophoresis to show size distribution. Fragments are then immunoprecipitated with an antibody that recognizes the protein of interest, allowing enrichment of the immunoprecipitate (IP) of a given region in the genome. DNA bound to the protein of interest is isolated and fragments bound are released in a process called de-crosslinking, from which the fragments can be purified. The samples are compared to the input sample, which has not been exposed to antibodies. This comparison is the basic measurement of ChIP. It is defined as IP efficiency, i.e. the amount of DNA in the IP sample compared to the input sample. The result of this comparison gives the fold enrichment over the control regions in the genome. Q-PCR based ChIP (Study II) analyzes the individual regions and compares IP DNA versus DNA input. In large-scale studies (Study III), the samples are prepared for further analysis such as microarrays or high-throughput sequencing, by creating a library and amplifying them using PCR. Both approaches will be covered in the sections below.

3.1.20 ChIP coupled to q-PCR (Study II)

ChIP was performed using the commercial kit from Diagenode, LowCell#Kit according to the manufacturer's instructions. Briefly, cells were fixed in 4% PFA at room temperature and the reaction was stopped using 0.14M glycine. The cells were then sonicated for 10 cycles on the BioRuptor at MAX output 30"sec on/30"sec off and centrifuged. Magnetic beads and antibody were incubated for 2 hours at 4^{o}C, fragments of DNA were added accordingly and samples for input were saved separately. Beads, antibody, and DNA fragments were incubated overnight at 4^{o}C. The following day, the magnetic beads were washed and captured. DNA was isolated from both IP DNA and input DNA, centrifuged, and analyzed with q-PCR. The levels of histone acetylation and methylation were evaluated with fold enrichment over DNA input.

3.1.21 ChIP-sequencing and library preparation (Study III)

ChIP was performed according to a protocol using nProtein A sepharose 4 fast flow beads. Briefly, the samples were fixed in 1% PFA for 10 minutes at room temperature and reaction was stopped using 0.25M glycine. The cells were sonicated for 7 cycles on the BioRuptor at MAX output 30"sec on/30"sec off and centrifuged. Samples were de-cross linked for 2 hours and then purified. Beads were washed and centrifuged, added to samples and incubated for 2-4 hours at 4^{o}C. Following incubation, beads + samples were washed and both IP DNA and input DNA were eluted. Samples were purified and analyzed with q-PCR. The levels of histone acetylation and methylation were evaluated with fold enrichment over DNA input. Libraries were made with NEXTflex ChIP-seq kit (BIOO Scientific, 5143-02) and barcoded with NEXTflex ChIP-seq barcodes (BIOO Scientific, 514-121) according to the manufacturer's protocol. Quality, size of fragment, and integrity of the library was analyzed with Agilent 2100 Bioanalyzer. Following sequencing, the resulting reads were aligned to a reference genome or reference transcripts. This was done to produce a genome-wide transcription map consisting of transcriptional structure and level of expression for each individual gene of interest.

3.1.22 Methylated DNA precipitation (MeDIP)

In epigenetics, identification of DNA methylation patterns is a common technique and used routinely in the lab. DNA methylation can silence genes and is important in disease development (157, 218). The sequencing methods available for DNA methylation analysis are mainly classified into;

i. Bisulfite sequencing such as whole genome bisulfite sequencing. DNA is converted with sodium bisulfite, which converts unmethylated cytosine to uracil that appears as thymine in sequencing reads, 5mC and 5hmC are protected from conversion and are both read as cytosine.
ii. Enrichment based methods, such as enrichment of either 5mC or 5hmC with specific antibodies. Samples can then be prepared for sequencing after library preparation.

Many array-based methods cannot separate the two methylation states, 5mC and 5hmC, from each other (219). 5hmC has been associated with activation in stem cell systems (220, 221) whereas 5mC has been shown to be present at gene promoters associated with transcriptional repression (147). We have therefore in study III used the enrichment-based method MeDIP, where an antibody against 5mC is used.

3.1.23 MeDIP-sequencing and library preparation (Study III)

MeDIP was performed using a commercial kit, NEXTflex^{TM} Methyl-seq 1 kit from BiooScientific according to manufacturers protcol with some minor modifications. Adaptors used were from BiooScientific, NEXTflex^{TM} DNA Barcodes. Briefly, DNA was isolated, and samples were sonicated for 50 cycles using the BioRuptor at MIN output 15 sec"on/15"sec off. Fragmentation was controlled with gel electrophoresis giving a fragmentation size between 200-400 base pairs. After sonication, part A of library preparation followed, which includes end repair, 3'-dA tailing and adapter ligation. For this we used 1µg of fragmented DNA as input material. After adapter ligation, MeDIP was performed using MagMeDIP kit from Diagenode following the manufacturer's protocol. The kit uses 5mC antibody against all regions in the genome and captures them with magnetic beads. A quality control to assess the efficiency of the MeDIP was done using q-PCR. We used primers

supplied in the kit, targeting GAPDH TSS and TSH2B. We compared the fold enrichment of methylation by comparing it to GAPDH regions, which should be low in methylated regions. After q-PCR validation of MeDIP, we continued with library preparation part B. The MeDIP samples were amplified using PCR, followed by library size selection to cut fragments down to sizes ranging between 300-700 base pairs. Quality, size of fragment, and integrity of the library was analyzed with Agilent 2100 Bioanalyzer. Samples were pooled on 3 separate lanes on an Illumna Hi-seq machine using single-reads. Following sequencing, the resulting reads were aligned to a reference genome or reference transcripts, and used to produce a genome-wide transcription map consisting of transcriptional structure and level of expression for each individual gene of interest.

3.1.24 RNA-sequencing (RNA-seq)

RNA-seq uses deep-sequencing technologies to map the transcriptome of selected cell populations. The transcriptome is the complete set of transcripts and the quantity of those transcripts within a cell. There are numerous ways to analyze and quantify the transcriptome:

i. Hybridization methods typically involves fluorescently labelled cDNA with custom-made microarray with a defined set of genes, or commercial high-density oligo microarrays.
ii. Sequence-based approaches directly determine the cDNA sequence. With the development of novel high-throughput sequencing methods, transcriptomes can be mapped and quantified genome-wide.

Following sequencing, the resulting reads are aligned to a reference genome or reference transcripts, to produce a genome-wide transcription map.

3.1.25 RNA-sequencing and library preparation

A commercial RNA-seq kit was used from Epicentre according to the manufacturer's protocol with minor modifications. Briefly, total RNA was purified using an RNeasy kit from Qiagen, and an input material of 20 ng of RNA was used. RNA populations were converted to cDNA in a 2-step manner and the cDNA was then tagged on both ends using indexes from Epicentre. All samples were amplified using PCR, and then size selected using AMPure XP magnetic beads, with size ranging between 300-800 base pairs. Quality, quantification and integrity of the library were analyzed with Agilent 2100 Bioanalyzer and Qubit. Samples were pooled and then sequenced on 3 separate lanes on the Illumna Hi-seq machine using single-read end primers from TruSeq Dual Index Sequencing Primer Box.

3.1.26 Statistical analyses (Study I-II)

For study I all data was presented ± standard error of mean using Students t-test (2 group comparison). P-values of less than 0.05 were considered to be significant. For study II the different passaging methods were compared using one-way repeated measures analysis of variance (one –way ANOVA), with the software Sigmaplot v.11.0. One-way ANOVA is used to test for differences in 3 groups or more using F-distribution.

4 RESULTS AND DISCUSSION

4.1 **Study I**

Previous studies of the ECM protein OSAD during tooth and bone development have shown its importance for mineralization. In the present study, we aimed to expand the understanding of the roles of ECM proteins in basic biomineralization. We have now provided new data on the involvement of OSAD in tooth development, and suggest a possible use of OSAD as a marker for mature osteoblasts and calcification.

The tooth is good model to study mineralization, as it consists of different layers of hard tissue, each with a specific degree of calcification. Furthermore, the building blocks of dentin are very similar to those of bone; mineralized matrix, collagen fibrils, and a wide range of ECM proteins, including the non-collagenous proteins. In particular, the predentin compartment is an excellent model for biomineralization. Here this process can be studied in a refined mode, without the ongoing remodeling that takes place during development in bone.

Consequently, in study I, incisors and molar teeth of mice at stages E15, 17, NB, and adult (>3 months) were used to map the dynamic distribution pattern of OSAD throughout developmental calcification. We provided detailed data on the quantitative distribution of OSAD using iEM, and further performed an analysis of OSAD localization as compared to other known SLRP's involved in bone and tooth development. This study provided a novel analysis of the localization pattern for OSAD during the different stages of tooth formation, and also confirmed its role in hard tissue mineralization. We used a highly specific OSAD antibody, and characterization with Western blot demonstrated that it could detect all glycosylated forms of OSAD. This is crucial, since SLRPs are known to be proteolytically processed during dentinogenesis (54).

Results from immunohistochemistry and iEM demonstrated that OSAD was localized to the predentin layer. However, the first sign of OSAD expression was not observed until the very early stages of dentinogenesis. Once initiated, the expression of predentin-dentin OSAD remained throughout development and was also seen in the mature erupted tooth. Biochemical, histochemical and ultrastructural studies have shown that SLRPs and GAG chains are differently distributed during mineralizing in the tooth, from the odontoblast cell layer to predentin, the predentin/dentin interface and dentin (52, 54, 58, 85). In accordance with previous findings, we did not detect OSAD expression in odontoblasts or in the enamel matrix (67). Comparisons of OSAD with that of other non-collagenous proteins FMD, BGN and DCN, using LM immunohistochemistry, showed that OSAD expression coincided chronologically with the expression of both BGN and FMD, whereas DCN was seen at a much earlier stage of tooth formation. OSAD was confined to the predentin and showed an accumulation towards the mineralization front. This was in difference to DCN, which was detected throughout the entire predentin. BGN and FMD, on the other hand, were found in close proximity to the odontoblast layer. We further investigated the OSAD subcellular distribution in tooth compartments using iEM. Using this technique, we have, to our knowledge, for the first time conclusively shown that there is a gradient of OSAD localization in the predentin layer, with an accumulation of OSAD adjacent to the mineralization front.

Our results support the notion that OSAD contributes to the pool of other KS-GAG chains near the border where calcification commences (85). Across this gradient, GAG chains of certain NCPs become closely associated with collagen fibers, allowing for a more defined fibrillogenesis to take place (86). Ultrastructurally, OSAD was found in the immediate vicinity of collagen fibers in the predentin and dentin layers of d5 mice, which supports the

notion that OSAD may be involved in collagen fibrillogenesis (67). However, whether or not OSAD binds to collagen fiber in the same manner as other SLRPs, such as BGN, DCN, and FMD remains to be functionally determined.

For functional studies of OSAD, we turned to an osteoblast model of mineralization, since the native dentin-producing odontoblasts de-differentiate and change phenotype *in vitro*. The results from the *in vitro* osteogenic model exhibited the expression patterns of OSAD during osteoblastic maturation. At the very early stages of proliferation and differentiation (d3 and d7), OSAD was expressed at very low levels, and the same low expression was seen for the other SLRPs investigated. The immature and unmineralized matrix at these early stages did not show signs of calcium deposits when stained with Alizarin Red S. Once ECM maturation and mineralization was initiated, as observed by the positive Alizarin Red S staining for calcium deposits by d 14 and d21, the levels of OSAD increased nearly 10-fold. OSAD thus followed the same patterns displayed by BGN, DCN and FMD, all of which have been reported to have a role in mineralization and matrix maturation (54).

From our studies we conclude that OSAD can indeed be considered as a marker for mineralizing cells. The expression patterns for DCN and FMD were similar to that of OSAD, since they increased with matrix maturation and mineralization. Taken together, these results underpin the essential roles of SLRPs in general and OSAD in particular in mineralization and demonstrate that without these proteins full mineralization cannot occur. The primary role of predentin SLRPs is to facilitate the organization of the ECM collagen matrix, and to direct hydroxyapatite binding and crystal growth (54), but the exact roles of KS-SLRPs need further investigations. Functional studies of OSAD interactions could be elucidated by studies in OSAD-mutated mice models, but unfortunately such models have still not been generated.

4.2 **Study II**

Various passaging techniques have been established in order to propagate PSC in a straightforward and effective way. Mechanical passaging involves scalpels or cell scrapers to “cut and paste” small colonies from one plate to another (134). Enzymatic passaging utilizes enzymatic or enzyme-free dissociation reagents to obtain single cells. Mechanical passaging is time-consuming, laborious and does not yield high numbers of cells. Consequently, single cell dissociation techniques are favored. Unfortunately, enzymatic dissociation of PSC disrupts cell-cell interactions, causing death of single cells. Rho-associated coiled-coil kinase (ROCK) inhibitor (ROCKi, Y-27632) has been used for single cell passaging, and can save single passages cells up to 27% (142). The molecular functions and effects of ROCKi on cells during passaging are not clearly understood. In this study, we wanted to highlight the importance of how PSCs are managed in the laboratory and shed light on ROCKi-mediated effects on the cells.

With regard to morphology of hESCs, we found that there were clearcut differences between mechanically and enzymatically (with or without addition of ROCKi) passaged cells. The mechanically passaged cells displayed smooth and sharp borders, which are typical characteristics for hESCs, and needed to be passaged with intervals of 4-5 days. Cells split enzymatically (pre-treated with ROCKi) produced more colonies in a shorter time (2-3 days), as compared to cells that were split mechanically. The cells that were split enzymatically (no ROCKi) grew slower, and generated fewer colonies when compared to the cells pre-treated with ROCKi. This could be perhaps explained by the fact that without ROCKi, the cells lost their cell-to-cell attachments, which led to some degree of apoptosis

(142). The mechanically treated cells and those treated with ROCKi formed colonies and cell death was at a minimum.

Next, we wanted to know if the augmented growth of the ROCKi-treated cells was due to a true propagation of pluripotent cells and not a result of clonal selection for cells with better capacity for growth. Cells from all three conditions were stained for stress fibers, as ROCKi has been indicated in apoptosis by controlling cell contraction (141). We found that mechanically and enzymatically passaged cells showed markers for stress fibers, as seen by the organized and dynamic actin filaments. However, cells treated with ROCKi had less stress fibers, indicating that ROCKi reduces the amount of stress caused by passaging. Furthermore, we stained the cells for Nanog to confirm their pluripotent status, and found them all to be positive.

Following these stainings, we performed RT-qPCR to examine the expression levels of the pluripotency markers Oct4, Nanog, Sox2, and Klf4. We found a significant decrease in gene expression of all the pluripotency markers using enzymatically passaging methods, as compared to mechanically dissociated cells. The loss of the pluripotency markers might indicate that the cells were assuming cancerogenic properties. However, c-MYC gene expression was not detected in any of the cells.

In order to explore if the decreased mRNA levels of these markers indicated an effect on the protein level, we labeled the cells with antibodies against Oct4, Nanog, and Sox2 for all passaging techniques. We found them to be immunopositive for all markers, and in addition, FACS analysis was positive for the pluripotent marker TRA-2-54. These results confirmed that the cells remained in a pluripotent state.

The differences seen on a gene expression level did not indicate an epigenetic regulation, as judged by observations of the histones H3K9me3, H3K4me3, and H3K27me3. Due to an increase in sizes of cell nuclei, we speculated that there might be a general effect on the chromatin structure, and thus an involvement of H4K16ac, as it has been implicated in nucleosome organization (222). However, we did not see any differences in modification of this histone that could be related to any of the different passaging techniques. These results might suggest that other important chromatin modifications control the pluripotency gene expressions that we saw at mRNA levels. Furthermore, investigating the DNA methylation status of these cells could also give clues as to why the levels of the pluripotency markers were decreased. DNA methylation combined with chromatin modifications in PSCs regulate gene expression and is also responsible for tightly regulating transcription upon differentiation (185, 186, 223). Methylated CpG islands in PSCs are generally present at promoters of repressed genes (185, 186), and for that reason it would valuable to continue the investigations from the present study with DNA methylation analysis or involvement of miRNA regulation.

Passaging and treating cells with ROCKi is an easy and effective way to propagate and handle large number of cells. However, we show here that there are considerable changes on the mRNA levels of pluripotency markers with this method, and we also demonstrate that morphology, nuclear size and actin staining of cells might differ in comparison with other handling techniques. Despite this, we found that all these effects were reversible when cells were returned to mechanical split. Our findings demonstrate, in general, the importance of the passaging technique when cultivating PSC, and in particular that this technique has to be taken into account when comparing experimental results from different studies that deal with similar or identical PSC research issues.

4.3 **Study III**

Study III aimed at characterizing the molecular mechanisms behind the clinical phenotypes seen in PIG-T patients, using iPSCs. The PIG-T gene belongs to a family of approximately 26 genes, involved in producing mature GPI anchors for more than 150 membrane proteins (224). iPSC lines from 3 affected children were compared with a healthy mother and an unrelated healthy control iPSC line. Although the main clinical features of the PIG-T mutation are neurological, we sought to find the molecular basis for the distinct facial and skeletal malformations displayed by these patients (225).

From our RNA-seq screen, we found that more than one hundred genes were differentially regulated in the sick children, when compared to control iPSC lines. Among these, 4 genes important in bone formation and skeletal development were identified, and these were shown to be significantly downregulated. The genes were *OPN*, *ACVR1*, *MMP2*, and *FOXO1*, all validated using RT-qPCR. Previously, deregulation in any of these four genes has been shown to lead to phenotypes similar to those seen in the sick children in the present study (226-230).

It has been reported that individuals with genetic mutations that cause intellectual disability often display dysmorphic facial features (231, 232) and are at increased risk for osteoporosis (233, 234). This coincides well with the patients seen in our study. One of the significantly downregulated genes, *OPN,* codes for an acidic glycoprotein secreted by mature osteoblasts and osteocytes. OPN binds to integrin's and enables bone cells to adhere to mineralized matrix (235, 236). We found indications of an epigenetic regulation of this gene in of the children. An increase of the repressive histone mark H3K27me3 was observed when compared to healthy iPSC controls. OPN null mice have a resistance to bone loss but this is due to impaired osteoclast function, hence an impaired regulation of bone homeostasis (230, 237). Impaired bone homeostasis correlates well with the phenotype seen in the children in the present study, indicating that their condition may somehow be linked to a malfunction of the OPN gene and subsequently increased osteoclast formation, causing the osteopenic state. Furthermore, we found that MMP2 levels in PIG-T mutated cell lines were downregulated, as compared to healthy control iPSC. Mice with an MMP2 null mutation display craniofacial defects and osteopenia (228), as well as weakened bone and a decrease in mineralization (238) – again the same phenotypes as seen in the children here, suggesting a contributing role for MMP2 as well.

FOXO1 is another gene important in skeletogenesis, being necessary during the first phases of mesenchymal cell differentiation into osteoblasts *in vitro* (229). We found decreased levels of *FOXO1*, and we also noted increased DNA methylation and a decrease of the active histone mark H3K4me3, in the children compared to healthy iPSC controls. The skeletal defects seen in the children at a very early stage might be explained by the disturbances in FOXO1 function early in mesenchymal cell differentiation into osteoblasts.

The decreased level of *ACVR1* mRNA expression was highly correlated with decreased levels of H3K4me3 in the patients, compared to iPSC controls. No change in the levels of the repressive histone mark H3K27me3 was noted, but we did find higher levels of DNA methylation at the ACVR1 gene promoter in the patients, indicative of epigenetic repression of the gene. ACVR1 is essential for normal mesoderm formation and null mice die before they are born (227). Interestingly we found increased levels of ACVR1 in the healthy mother, accompanied by a decrease in H3K4me3 compared to iPSC controls. These findings indicate that the levels seen in the sick children are insufficient for the receptor ACVR1 to function as

an anchorage for BMP7, and thus proper bone formation through canonical Wnt signaling is flawed, whereas the levels of ACVR1 in the mother allows for proper bone formation.

To conclude, we have provided evidence of 4 novel genes that are differentially regulated on mRNA and epigenetic levels, which can have a causative role in the phenotypes seen in PIG-T mutant patient cells. In the original study on the PIG-T mutation by Kvarnung et al (225) it was suggested that (tissue-nonspecific alkaline phosphatase) TNALP, a GPI anchored enzyme essential for bone mineralization (39, 239), was the cause of the phenotypes seen in the patients (225). When TNALP cannot bind to GPI it becomes elevated in serum and causes softening of bone and hypomineralization (240). Thus it was hypothesized that decreased levels of TNALP caused the skeletal malformations, although this was not investigated. In our study, TNALP did not appear as an interesting putative causative target gene. Instead, we propose more prominent roles of the four candidate genes we found here for the bone and tooth defects seen in the patients. We further propose that they can be useful as diagnostic markers in bone diseases and skeletal malformations in other conditions.

The patient iPSCs have been thoroughly characterized before differentiation into the osteoblastic lineage. We believe that the difference in mRNA and epigenetic levels in the pluripotent state of these cells, when compared to healthy iPSCs, might indicate a deregulation that is propagated during disease development. Furthermore, the overall low abundance of changes in mRNA and epigenetic markers might be responsible for the phenotypes seen in the patients. This “primed state” of the iPSC lines could be explained by a residual epigenetic memory in the patient lines compared to the healthy control lines. Epigenetic memory has shown to have role in differentiation potential and general differences between PSC lines (155, 156). Changes in mRNA expression, which cannot be explained by neither DNA methylation nor histone modification, could potentially be answered by changes in miRNA involvement during embryogenesis and early development. In line with this notion the role of miRNA has been shown to be highly important in bone development (43-46).

Results from our study are of high interest and might possibly be the explanation for the phenotypes in these children, and suggest a “primed state” of the disease iPSC in the children. However, we need to validate our findings with functional *in vitro* studies to determine the function of the genes found. As a first step cells would be differentiated into the osteoblastic lineage and from there, each gene found from our study could be silenced, overexpressed or rescued in *in vitro* cultures using shRNA’s or CRISPR systems. There is a number of potential clinical applications for genome editing in PSCs, including knocking out or knocking in a gene of interest, and also correcting a mutation in a patient-specific iPSC line. CRISPR systems have been shown to generate genome editing in human PSC (241, 242). This approach can be used to generate genetically modified cell lines with sequence specific nucleases.

Overall, the genes found to be downregulated in our study could play a functional role in the patient phenotypes observed. Although additional studies are needed to confirm our findings we believe that these genes are of high interest in disturbed bone development, and might play a potential role in other bone developmental defects as well.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Reconstruction of bone is an enormous health care problem, further complicated by increasing therapy cost and an aging population (243, 244). Skeletal defects can result from trauma, disease, or congenital abnormalities, and can compromise quality of life and normal function of the body. The currently available materials to reconstruct skeletal defects include bone from the patient (autologous bone), bone from a donor (allogeneic bone), and also inorganic materials (245). Autologous bone grafts are usually obtained from the iliac crest and do not cause an immune rejection, however these grafts have been associated with donor site morbidity and inflammation (245). Allogeneic bone is usually demineralized bone from a deceased body and has poor quality with a higher degree of bone resorption than newly formed bone. The inorganic materials carry a high risk infections, and can cause repeated painful surgeries (246). Hence, there is a high demand of functional bone to be used in the clinics for bone regeneration and more research in how to develop such bone is needed.

The ultimate goal with this thesis work was, through different approaches, to expand the knowledge in basic bone biology and bone development, using *in vitro* cell culture model systems. To gain a wider understanding of biomineralization, we studied the roles of some of the constituents of the ECM of calcified tissue, such as members of the SLRP family. This study showed that the KS-SLRP OSAD is strictly confined to the mineralization front in the tooth, and that its expression coincides with that of other known markers for mineralization. Furthermore, OSAD proved to be an excellent marker of ECM maturation in an *in vitro* model system of osteogenesis, where its expression coincided with that of other known markers for mineralization. With this new knowledge of bone biology at the level of ECM proteins, which elucidates some of the fundamentals of mineralization, we turned to stem cell cells as tool for further studies. As a preparation for subsequent *in vitro* stem cell applications in bone biology, we began by highlighting the importance of cell culture techniques when growing PSC in the laboratory.

We have underlined the importance of handling of PSC in the laboratory, to keep the cells from undergoing spontaneous differentiation and allow correct settings for experiments, differentiation and transplantations. In study II we showed that cells passaged mechanically and enzymatically without any ROCKi treatment displayed stress activation, indicated by organized actin filaments. Cells treated with ROCKi had a different morphology but did not display any signs of stress fibers, and although they showed decreased mRNA levels of pluripotent markers, they were still pluripotent as judged by protein expression. Furthermore, we could not detect any signs of epigenetic regulation that might be responsible for the changes in pluripotency markers. These results indicate that the handling of stem cells in experimental setups is crucial, and needs to be thoroughly examined when experimental results from different studies are compared.

A human congenital skeletal malformation is difficult to study *in vivo*, since progress of the syndrome usually is initiated already before birth. To understand and study the basic molecular mechanisms underlying these disease states, PSC stem cell model systems are of great use. Combining the knowledge from study I and II, we used an iPSC based model system to study patients with a type of congenital skeletal malformations caused by a mutation in the PIG-T gene. We mapped chromatin modifications and DNA methylation patterns, to illuminate the importance of epigenetics in the disease, and detected osteogenesis-related genes in RNA-seq data that seem to be involved in the bone phenotype of this syndrome. Furthermore, these genes showed signs of epigenetic regulation.

Cell therapy using stem cells that adopt a mineralizing phenotype holds great potential for regeneration of calcified tissues such as bone and teeth. Stem cells can provide an unlimited

number of cells that can be used for bone differentiation. The mechanisms governing stem cell bone formation are, however, complex and involve many factors. Our study has generated new knowledge of basic bone biology, handling of stem cells in *in vitro* culture systems and the complex molecular mechanisms associated with normal and impaired bone development. We believe that we have provided the field of bone tissue engineering with new insights into basic bone biology (study I), handling of PSC in culture for future use osteoblastic differentiation (study II), and that we have shed light on important and novel bone development-related genes regulated on epigenetic level (study III).

Future perspectives

Overall, great progress has been made in the field of stem cell-based bone regeneration. PSC are promising cell sources, but there are dire needs of reliable laboratory protocols to ensure that PSC can differentiate into functional osteoblast *in vivo* and retain their capacity to produce bone over time. Another major issue with PSC is the need to quench their tumorigenic potential and to safeguard from unwanted immune responses. Still, having considered this, the field of bone regeneration is promising, and functional osteoblasts derived from stem cells may eventually come into clinical use.

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